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13. ABSTRACT (Maximum 200 words) Using stable and radioisotope tracers, and computer simulation of resulting isotopic tracer data, the pathway of synthesis of 3-dimethylsulfoniopropionate (DMSP) was found to proceed via a hitherto unidentified route from methionine. The intermediates are: 4-methylthio-2-oxobutyrate (MTOB), 4-methylthio-2-hydroxybutyrate (MTHB) and 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB). All algae tested (including <i>Enteromorpha intestinalis</i> , <i>Tetraselmis</i> sp., <i>Melosira nummuloides</i> and <i>Emiliania huxleyi</i>) metabolized supplied ^{35}S -methionine to ^{35}S -MTOB, ^{35}S -MTHB, ^{35}S -DMSHB and ^{35}S -DMSP, and metabolized supplied ^{35}S -DMSHB to ^{35}S -DMSP. Enzymes catalyzing the first three enzymes of the pathway were characterized. These include a methionine:2-oxoglutarate-dependent aminotransferase with a high affinity for methionine, an NADPH-dependent reductase, and an S-methyltransferase. In certain phytoplankton, synthesis of the first intermediate may be augmented by a methionine:pyruvate-dependent aminotransferase and methionine oxidase. DMSP (a sulfur-containing osmolyte) and glycine betaine (a nitrogen-containing osmolyte) are reciprocally regulated by nitrogen supply in <i>Tetraselmis</i> . Accumulation of DMSP under nitrogen-limitation may in part involve derepression of methionine aminotransferase and methionine oxidase. No evidence for the involvement of S-methylmethionine or DMSP-aldehyde as pathway intermediates was found in marine algae. The pathway of DMSP synthesis in marine algae is therefore different from that found in higher plants.				
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To disseminate methods for computer simulation of isotopic tracer data, a computer modeling www site (acknowledging ONR grant support) has been established:

<http://www.hort.purdue.edu/cfpesp/models/models.htm>

This site includes interactive Java applets and downloadable Visual Basic programs that simulate the flux of ^{35}S -Met to ^{35}S -DMSP in Enteromorpha intestinalis, and ^{35}S -S-methylmethionine to ^{35}S -DMSP in the higher plant, Spartina alterniflora.

CONCLUSIONS: The key intermediates in the DMSP synthesis pathway from methionine (MTOB, MTHB and DMSHB) are the same in four diverse groups of marine algae tested, although species differ with respect to the relative contributions of 2-oxoglutarate- and pyruvate-dependent Met aminotransferases and Met oxidase in MTOB synthesis. The pathway of synthesis of DMSP in marine algae is completely different from that in higher plants, where S-methylmethionine and DMSP-aldehyde are key intermediates. The occurrence of a high affinity methionine aminotransferase that is depressed by N starvation may begin to explain why DMSP accumulation is stimulated by N-deficiency in marine algae.

SIGNIFICANCE: The work has established a novel pathway of synthesis of DMSP in marine algae. DMSP produced in oceans by marine algae is recognized to be a precursor of atmospheric dimethylsulfide (DMS) that plays an important role in the global sulfur cycle. The identification of DMSHB as an intermediate in the algal pathway raises the possibility that this compound may also contribute to DMS emissions from marine algae. Methods for computer simulation of isotopic tracer data were developed and have been made available to the scientific community.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1. Gage, D.A., Rhodes, D., Nolte, K.D., Hicks, W.A., Leustek, T., Cooper, A.J.L., Hanson, A.D. (1997) A new route for synthesis of dimethylsulphoniopropionate in marine algae. *Nature* **387**: 891-894.
2. Islam, S. (1997) Characterization of the first step involved in DMSP production in marine algae. Master of Science Thesis, Purdue University, 97 pages.
3. Summers, P.S., Nolte, K.D., Cooper, A.J.L., Borgeas, H., Leustek, T., Rhodes, D., Hanson, A.D. (1998) Identification and stereospecificity of the first three enzymes of 3-dimethylsulfoniopropionate biosynthesis in a chlorophyte alga. *Plant Physiol.* **116**: 369-378.
4. Rhodes, D., Shachar-Hill, Y., McNeil, S.D., Hanson, A.D. (1999) Computer simulation of isotope labeling kinetics of metabolic networks: uses in interpreting precursor-product relationships, fluxes and compartmentation of pools. Presented at 1st Annual Plant Biochemistry and Molecular Biology Conference "Metabolic Networking in Plants", Iowa State University, April 24, 1999; p. 15. [copy attached].
5. Rhodes, D., Nadolska-Orczyk, A., Rich, P.J. (2000) Salinity, osmolytes and compatible solutes. In (A. Läuchli, U. Lüttge, eds.) "Salinity: Environment - Plants - Molecules", Kluwer Academic (In Press) [copy attached].

6. Rhodes, D., Islam, S., Rich, P.J., Nadolska-Orczyk, A. (2000)
Metabolism of methionine in Tetraselmis sp. Plant Physiol. (in final
stages of preparation).

FINAL REPORT

GRANT #: N00014-96-1-0366

PRINCIPAL INVESTIGATOR: Dr. David Rhodes

INSTITUTION: Purdue University

GRANT TITLE: Biosynthesis of 3-Dimethylsulfonipropionate in Marine Algae

AWARD PERIOD: 1 Jan 1996 - May 30 1999 (36 months plus 6 month no cost extension = 42 months total)

OBJECTIVE: To participate in a 4-investigator collaboration (Hanson, Gage, Leustek, Rhodes) to elucidate the pathway of 3-dimethylsulfonipropionate (DMSP) in marine algae, including identification of intermediates and enzymes of the pathway in the macroalgae Enteromorpha intestinalis, and three diverse marine phytoplankton species; Tetraselmis sp., Melosira nummuloides and Emiliania huxleyi. Develop quantitative models describing flux of through the pathway and its competing branches, and investigate regulation of the pathway.

APPROACH: In vivo stable and radioactive isotope tracer experiments in combination with various chromatographic (ion exchange, TLC, TLE) and mass spectrometric methods, were employed to identify and quantify the intermediates and elucidate precursor-product relationships in the DMSP synthesis pathway. Computer simulation was employed to quantify metabolic fluxes. Enzyme assays, using radioisotope labeled substrates, were used to identify enzymes mediating the reactions.

ACCOMPLISHMENTS: In collaboration with Hanson, Gage and Leustek, the pathway of synthesis of DMSP in Enteromorpha intestinalis, Tetraselmis sp., Melosira nummuloides, and Emiliania huxleyi was shown to proceed via the following common steps: methionine (Met) → 4-methylthio-2-oxobutyrate (MTOB) → 4-methylthio-2-hydroxybutyrate (MTHB) → 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) → 3-dimethylsulfoniopropionate (DMSP). All algae tested synthesized ³⁵S-MTOB, ³⁵S-MTHB, ³⁵S-DMSHB and ³⁵S-DMSP from supplied ³⁵S-Met, and metabolized supplied ³⁵S-DMSHB to ³⁵S-DMSP.

Computer modeling showed that the ³⁵S labeling kinetics of MTHB and DMSHB are quantitatively consistent with these compounds being intermediates in the metabolism of ³⁵S-Met to ³⁵S-DMSP in E. intestinalis, but may be compartmentalized between metabolically active and inactive (storage) pools. Modeling also showed that the first step must compete effectively with protein synthesis in vivo, suggesting that this step must have a high affinity for Met. Enzymes catalyzing the first three steps in the pathway were identified in E. intestinalis; a 2-oxoglutarate-dependent Met aminotransferase with a high affinity for Met (30 μM), an NADPH-dependent MTOB reductase, and an S-adenosylmethionine-dependent MTHB S-methyltransferase. Consistent with a 2-oxoglutarate-dependent aminotransferase at the head of the pathway in E. intestinalis, ¹⁵N-methionine was metabolized predominantly to ¹⁵N-glutamate in this species. In vivo ¹⁸O-labeling data indicate

that the final step (DMSHB \rightarrow DMSP) is catalyzed by an oxygenase in Enteromorpha, but the enzyme catalyzing this reaction was not characterized.

Certain of the marine phytoplankton were found to possess chromatographically distinct aminotransferase isoforms. Two separate 2-oxoglutarate-dependent Met aminotransferases were characterized in Tetraselmis, whereas only a single form of this enzyme was found in Emiliana and Melosira. However, distinct pyruvate and 2-oxoglutarate-dependent aminotransferases were found in Emiliana, with the former being induced in late stationary phase. A methionine oxidase that catalyzes MTOB synthesis from Met [Met + H₂O + O₂ \rightarrow MTOB + H₂O₂ + NH₃] was also detected in Melosira and Tetraselmis. In vivo ¹⁵N-Met and ¹⁵NH₃ labeling experiments (with and without the inhibitors of transamination and ammonia assimilation, aminooxyacetate and methionine sulfoximine, respectively) showed that Met is largely (67%) reversibly transaminated to glutamate in Tetraselmis, with the remainder (33%) either deaminated to NH₃ via the catalytic action of methionine oxidase (~26%) or methioninase (~7%) [Met \rightarrow 2-ketobutyrate + NH₃ + CH₃-SH]. The 2-ketobutyrate derived from the action of methioninase is rapidly converted to isoleucine. NH₃ generated in the methionine oxidase and methioninase reactions is reassimilated predominantly via the glutamine synthetase-glutamate synthase cycle (>95%), and in part via glutamate dehydrogenase (<5%). A double-labeling procedure (using methyl-CD₃, ¹⁴N-Met supplied to Tetraselmis cells pregrown on ¹⁵N-nitrate for several generations to fully label all N moieties with ¹⁵N) was developed for simultaneously determining aminotransferase, Met oxidase, methioninase and activated methyl cycle [CD₃-Met \rightarrow CD₃-S-adenosylmethionine \rightarrow S-adenosylhomocysteine \rightarrow homocysteine (+ CH₃-THF) \rightarrow CH₃-Met (+ THF)] fluxes in vivo. Computer modeling of the time-courses of changes in abundance of CD₃, ¹⁴N-Met, CH₃, ¹⁴N-Met, CD₃, ¹⁵N-Met and CH₃, ¹⁵N-Met species showed that the activated methyl cycle flux is approximately twice the Met aminotransferase flux in Tetraselmis.

Evidence was obtained that endogenous accumulation of Met inhibits flux to DMSP in Tetraselmis; this inhibition must occur at a step later than transamination/oxidation in the pathway, but earlier than the DMSHB \rightarrow DMSP step because DMSHB does not accumulate when the free Met pool is elevated. The precise mechanism of this inhibition has not been elucidated, but may involve inhibition of the MTHB S-methyltransferase by Met, or a product of Met metabolism (e.g. S-adenosylhomocysteine). Fluxes to DMSP and the nitrogenous osmolyte, glycine betaine, are reciprocally regulated by N supply in Tetraselmis. Stimulation of DMSP accumulation by N limitation may in part involve derepression of Met aminotransferases and Met oxidase, depletion of the cellular glutamate pool [favoring a shift in the equilibrium of the 2-oxoglutarate + Met \leftrightarrow Glu + MTOB aminotransferase reaction towards MTOB synthesis], and alleviation of the MTHB:S-methyltransferase from inhibition by Met (or a metabolite derived therefrom).

No evidence was found for the involvement of S-methylmethionine (SMM) or DMSP-aldehyde in DMSP synthesis in any of the marine algae tested. This contrasts to higher plants where DMSP synthesis proceeds via the following route: Met \rightarrow SMM \rightarrow DMSP-aldehyde \rightarrow DMSP.

A new route for synthesis of dimethylsulphoniopropionate in marine algae

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The 3-dimethylsulphoniopropionate (DMSP) produced by marine algae is the main biogenic precursor of atmospheric dimethylsulphide (DMS)¹⁻³. This biogenic DMS, formed by bacterial and algal degradation of DMSP^{4,5}, contributes about 1.5×10^{13} g of sulphur to the atmosphere annually³, and plays a major part in the global sulphur cycle, in cloud formation and potentially in climate regulation^{1,3}. Although DMSP biosynthesis has been partially elucidated in a higher plant^{6,7}, nothing is known about how algae make DMSP except that the whole molecule is derived from methionine⁸⁻¹². Here we use *in vivo* isotope labelling to demonstrate that DMSP synthesis in the green macroalga *Enteromorpha intestinalis* proceeds by a route entirely distinct from that in higher plants. From methionine, the steps are transamination, reduction and S-methylation to give the novel sulphonium compound 4-dimethylsulphonio-2-hydroxybutyrate (DMSHB), which is oxidatively decarboxylated to DMSP. The key

intermediate DMSHB was also identified in three diverse phytoplankton species, indicating that the same pathway operates in other algal classes that are important sources of DMS. The fact that a transamination initiates this pathway could help explain how algal DMSP (and thereby DMS) production is enhanced by nitrogen deficiency¹².

Many marine phytoplankton species and intertidal macroalgae accumulate DMSP as an osmolyte and cryoprotectant, particularly when salinity is high and nitrogen is limiting^{2,12,13}. We investigated DMSP synthesis first in the green macroalga *Enteromorpha intestinalis*, which is rich in DMSP ($\sim 20 \mu\text{mol g}^{-1}$ fresh weight) when grown in normal sea water. To identify biosynthetic intermediates between methionine (Met) and DMSP, we followed the metabolism of a small dose of ^{35}S -methionine. The main fates of ^{35}S Met were conversion to DMSP and incorporation into protein (Fig. 1a). Two stable compounds acquired ^{35}S rapidly and lost it as the ^{35}S Met dose was depleted, as expected for DMSP pathway intermediates; these were 4-methylthio-2-hydroxybutyrate (MTHB) and its S-methylated derivative DMSHB, a new natural product (Fig. 1b). The most plausible route from Met to these compounds is via the unstable 2-oxo acid, 4-methylthio-2-oxobutyrate (MTOB). We therefore tested for ^{35}S incorporation into MTOB by using a gentle extraction method (Table 1, method A) or by converting it to a stable derivative (Table 1, method B). Both approaches confirmed that MTOB acquired and lost ^{35}S in parallel with MTHB.

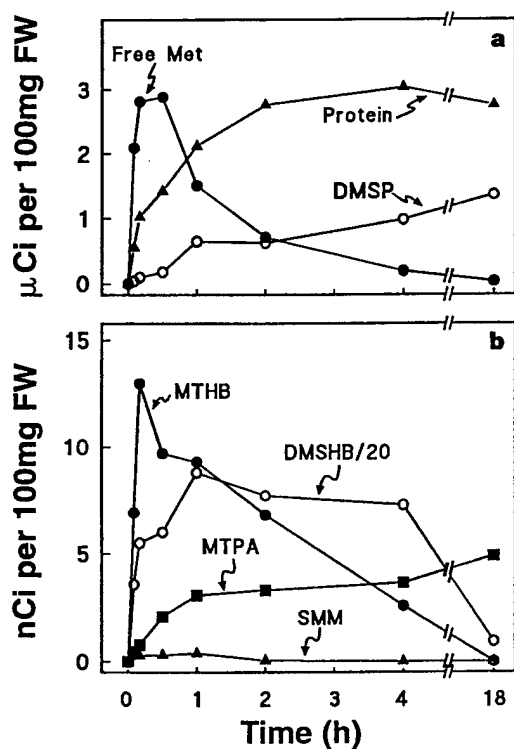


Figure 1 Labelling kinetics of selected small molecules and protein following administration of a tracer ^{35}S Met dose to *E. intestinalis*. Batches of fronds (100 mg fresh weight, FW) were supplied with $5 \mu\text{Ci}$ (0.5 nmol) ^{35}S Met. **a**, Free Met, DMSP and protein; **b**, Potential DMSP pathway intermediates; DMSHB data points are reduced by a factor of 20; MTPA, 3-methylthiopropylamine. No label ($\leq 0.5 \text{ nCi per } 100 \text{ mg}$) was detected in 3-dimethylsulphoniopropionaldehyde.

Computer modelling¹⁴ confirmed that the labelling kinetics of MTOB, MTHB and DMSHB were quantitatively consistent with roles as intermediates.

None of the other compounds monitored had labelling kinetics expected of intermediates in DMSP synthesis. S-Methylmethionine (SMM) and 3-dimethylsulphoniopropionaldehyde, both known to be DMSP synthesis intermediates in higher plants^{6,7}, acquired little or no ^{35}S (Fig. 1b). 3-Methylthiopropylamine, which has been proposed to be an intermediate in algae¹¹, labelled as would be expected for a minor end product (Fig. 1b). Labelling of another hypothetical intermediate, 3-methylthiopropionate^{8,11} varied from undetectable to comparable to that of MTOB in experiments with different lots of *E. intestinalis*. This can be attributed to a catabolic route involving oxidative decarboxylation of MTOB, which occurs in algae and other plants that do not contain DMSP, as well as in animals, and whose activity can vary with nutritional status^{9,15-17}.

These data support the pathway $\text{Met} \rightarrow \text{MTOB} \rightarrow \text{MTHB} \rightarrow \text{DMSHB} \rightarrow \text{DMSP}$, but are not consistent with a route involving SMM. To test this, ^{35}S -labelled SMM, MTHB and DMSHB were supplied (Table 2). SMM was scarcely metabolized, providing additional evidence that it is not an intermediate. MTHB was mainly converted to methionine, which in turn was incorporated into protein—a fate of MTHB well known in other plants and in animals^{18,19}. Some ^{35}S from MTHB also entered DMSP, consistent with MTHB being an intermediate; however, an indirect route via

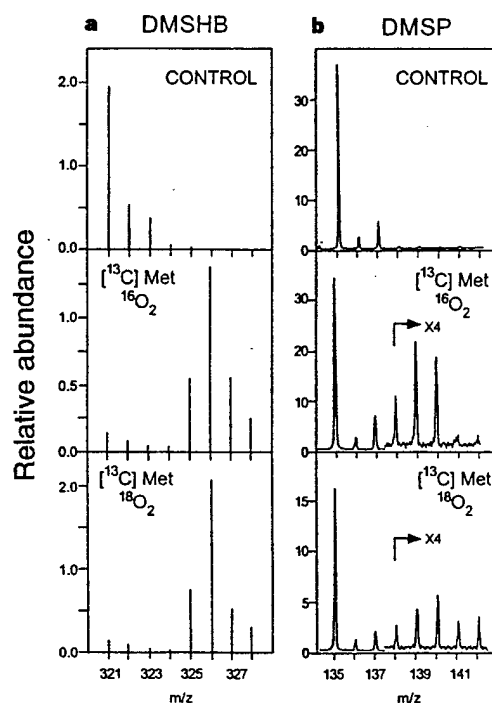


Figure 2 Evidence for conversion of DMSHB to DMSP by an oxygenase reaction in *E. intestinalis*. Fronds (100 mg) were incubated for 24 h with L-[U- $^{13}\text{C}_6$]Met (5 μmol) in flasks initially containing 21% $^{16}\text{O}_2$ or $^{18}\text{O}_2$ in N_2 ; controls received no Met and no $^{18}\text{O}_2$. **a**, SIM analysis of DMSHB. The peak areas in the control fit the expectation for natural-abundance C, S, Si and O isotopes. In the ^{13}C Met/ $^{16}\text{O}_2$ treatment, endogenous DMSHB was replaced by $^{13}\text{C}_4$ - and $^{13}\text{C}_5$ -labelled species (the S-demethylated derivatives of $^{13}\text{C}_5$ - and $^{13}\text{C}_6$ -DMSHB). Exposure to ^{18}O did not change this pattern, showing that the α -hydroxyl group does not originate from O_2 . **b**, FAB-MS analysis of DMSP. In the ^{13}C Met/ $^{16}\text{O}_2$ treatment, the peaks at m/z 139 and 140 are $^{13}\text{C}_4$ - and $^{13}\text{C}_5$ -DMSP. Under $^{18}\text{O}_2$, the signals at m/z 141 and 142 ($^{13}\text{C}_4$, ^{18}O - and $^{13}\text{C}_5$, ^{18}O -DMSP) show that 30–40% of the ^{13}C -labelled DMSP molecules contain an ^{18}O atom.

methionine cannot be excluded. As would be expected for a late intermediate in the pathway, DMSHB was metabolized efficiently to DMSP and to little else.

Stable isotope labelling was used to confirm and extend the radiotracer findings. We first verified the presence of the novel intermediate DMSHB and tested whether its conversion to DMSP involves an oxygenase reaction, perhaps analogous to that mediated by lactate oxidase²⁰. To do this, *E. intestinalis* was given [$U-^{13}C_5$]Met in an atmosphere containing $^{16}O_2$ or $^{18}O_2$. Gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM) revealed a small pool of DMSHB that became labelled with ^{13}C in five or six positions, but not with ^{18}O (Fig. 2a). Fast atom bombardment (FAB)-MS showed that most of the newly synthesized DMSP had four or five ^{13}C atoms and that 30–40% of this DMSP became labelled with ^{18}O (Fig. 2b). Note that recycling of the [$^{13}C_4$]homocysteine moiety formed in methylation reactions would yield Met (and hence DMSHB and DMSP) with an unlabelled methyl group. The presence of such Met was confirmed by GC-MS, and modelling¹⁴ showed the observed proportions of [$^{13}C_4$]- and [$^{13}C_5$]DMSP conformed to expectations for recycling of the [$^{13}C_4$]homocysteine moiety generated by the methylation step in DMSP synthesis. Also, photosynthetically generated $^{16}O_2$ lowers the intracellular abundance of $^{18}O_2$. The ^{13}C and ^{18}O data are thus fully consistent with a pathway in which the last step is an oxygenase-mediated oxidative decarboxylation of DMSHB.

Table 1 Metabolism of [^{35}S]Met by *E. intestinalis*

Extraction method	Incubation time (h)	^{35}S incorporation (nCi per 100 mg)		
		MTOB	MTHB	DMSHB
A	0.5	17.3	6.8	51
	4.0	5.2	1.4	22
B	0.5	18.9	3.4	110
	4.0	≤2.0	0.6	35

Fronds (50 or 100 mg) were labelled and analysed as for Fig. 1, apart from the extraction (see Methods). The fronds extracted by methods A and B came from separate samples of algae.

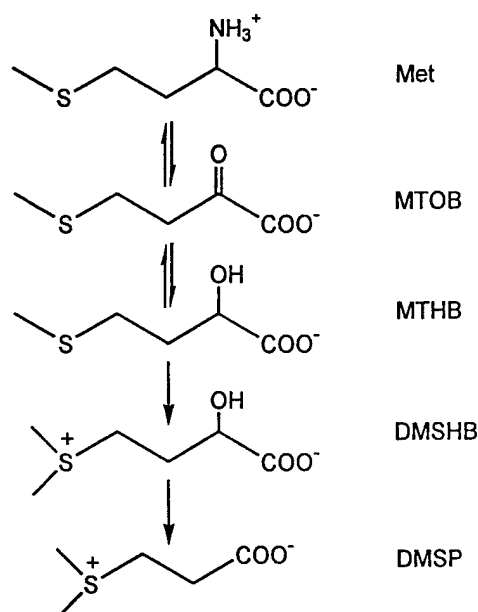


Figure 3 Proposed pathway of DMSP synthesis in *E. intestinalis* and other algae. The second step is shown as reversible because MTHB can be converted to Met (Table 2).

We also used stable isotope labelling to investigate the conversion of methionine to MTOB. *E. intestinalis* was given [^{15}N]Met (5 μ mol per 100 mg) and GC-MS was used to follow the labelling of amino acids. Glutamate acquired ^{15}N readily (7.0% abundance at 2 h), as did aspartate and alanine, but the amide group of glutamine did not (<1% abundance at 2 h). This is consistent with transamination of methionine, but not with oxidative deamination: the $^{15}NH_3$ from a deamination reaction would have led to labelling of glutamine amide via the action of glutamine synthetase²¹. The operation of glutamine synthetase in *E. intestinalis* was confirmed by supplying $^{15}NH_4^+$ (1 μ mol per 100 mg) and showing that glutamine amide nitrogen was rapidly labelled (42% ^{15}N abundance at 2 h). The conversion of MTHB to Met (Table 2) indicates that the Met \rightarrow MTOB step is reversible, which is consistent with transamination²².

Collectively, the data for the chlorophyte macroalgae *E. intestinalis* indicate that the pathway for synthesis of DMSP is that shown in Fig. 3. We tested for this pathway in marine planktonic species as these are major producers of DMSP and DMS^{1–3}. For this, we chose a prymnesiophyte (*Emiliania huxleyi*), a diatom (*Melosira nummuloides*) and a prasinophyte (*Tetraselmis* sp.). These algae all contained small pools of the key intermediate DMSHB which acquired label from [^{35}S]Met and lost it as the [^{35}S]Met was consumed (Fig. 4). All of them metabolized supplied [^{35}S]DMSHB to [^{35}S]DMSP (Fig. 4, inset). It is therefore likely that they have the same pathway as *E. intestinalis*.

Table 2 Uptake and metabolism of ^{35}S -precursors by *E. intestinalis*

Precursor	^{35}S uptake (nCi)	^{35}S incorporation (% of uptake)	
		DMSP	Protein Met
Met	454	6.2	58
SMM	451	≤0.2	≤1
MTHB	116	2.7	24
DMSHB	432	15.5	≤1

Data for Met are included for comparison. Fronds (100 mg) were incubated for 4 h with 500 nCi (1.1–1.4 nmol) of each compound. L-Isomers were supplied for all compounds, although these could have been racemized *in vivo*²³.

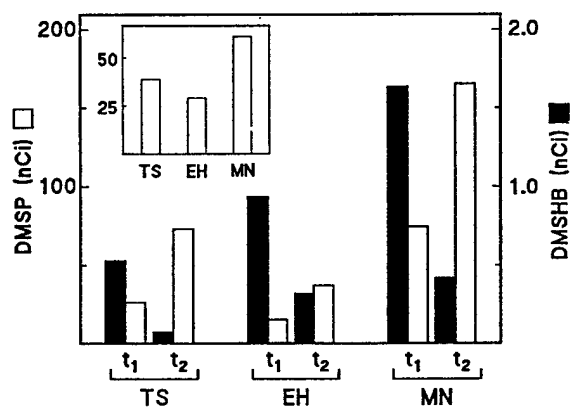


Figure 4 Evidence that DMSHB participates in DMSP synthesis in *Emiliania huxleyi* (EH), *Melosira nummuloides* (MN) and *Tetraselmis* sp. (TS). The main figure shows conversion of [^{35}S]Met to DMSP and DMSHB. Data are for 10^7 (EH and TS) or 10^6 cells (MN). Cultures were incubated with [^{35}S]Met (8–10 μ Ci; about 0.5 nmol) for a short time (t_1) to label heavily the pools of free Met and intermediates, and for a long time (t_2) to allow ^{35}S to chase from these pools. Times t_1 and t_2 (h), culture volumes (ml) and cell numbers were: EH, 1.5, 23, 12, 5×10^7 ; MN, 2.5, 23, 1.2, 5×10^6 ; TS, 0.25, 16, 25, 4.4×10^7 . The inset shows synthesis of [^{35}S]DMSP (nCi per 10^7 cells) from [^{35}S]DMSHB (2–3 μ Ci, 0.2–0.3 nmol). Incubation was for 17–23 h. Culture volumes (ml) and cell numbers were: EH, 10, 5.5×10^7 ; MN, 1.2, 5×10^6 ; TS, 10, 1.4×10^7 .

Our data establish a pathway for DMSP biosynthesis in marine algae. This pathway has no steps in common with that in higher plants, which proceeds via SMM and 3-dimethylsulphonio-propionaldehyde^{6,7}. DMSP biosynthesis must therefore have evolved independently at least twice. Our results have two other implications. The first stems from the finding that a transaminase reaction stands at the head of the DMSP pathway; this may help explain why nitrogen deficiency enhances DMSP production^{12,23,30}. Depletion of cellular amino acids would favour the transamination reaction, thereby promoting DMSP synthesis when nitrogen is limiting. Second, our results suggest that DMSP may not be the only precursor of the DMS produced by living algae: DMSHB is another potential precursor *in vivo*. In support of this possibility, we have obtained preliminary evidence for extensive catabolism of supplied DMSHB to DMS in *Tetraselmis* sp. and *E. huxleyi*. □

Methods

Algae. *E. intestinalis* was collected in Florida and kept in aerated sea water at 18 °C in continuous fluorescent light (photosynthetic photon flux density, 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). *M. nummuloides* (CCMP 482) and *Tetraselmis* sp. were cultured axenically at 25 °C in the above light regime in modified Goody's medium²⁴ with 1 mM NO₃⁻; for *M. nummuloides*, 0.1 mM Na₂SiO₃ was added and Tris omitted. *E. huxleyi* (CCMP 373) was grown in f/2 medium²⁵ in daylight at 22 °C.

Radiochemicals. L-[³⁵S]SMM was synthesized enzymatically from L-[³⁵S]Met²⁶ or by treating L-[³⁵S]Met with 250 mM methanol in 6 M HCl at 110 °C for 4 h (ref. 27), and converted to L-[³⁵S]DMSHB with HNO₂ (ref. 28). [³⁵S]MTOB was made from L-[³⁵S]Met using L-amino acid oxidase, and converted to L-[³⁵S]MTHB using L-lactic dehydrogenase and NADH; [³⁵S]methylthiopropionate was obtained as a byproduct. Compounds were purified by ion exchange and TLE^{6,7}.

Labelling conditions. *E. intestinalis* fronds (50 or 100 mg) were incubated in 0.5–2.5 ml sterile sea water; ¹⁸O labelling was carried out in 50-ml flasks. For phytoplankton species, labelled compounds were added to growing cultures. Incubation was at 18–21 °C for *E. intestinalis* and *E. huxleyi* and 25 °C for *Tetraselmis* and *M. nummuloides*, under fluorescent light and with gentle agitation. Uptake of ³⁵S was estimated from its disappearance from the medium.

Metabolite analysis. Most metabolites were isolated by methanol–chloroform–water extraction, ion exchange, TLE and TLC^{6,7}. As MTOB broke down in these procedures, forming MTP, these compounds were isolated using 0.1 M HCl followed by ether extraction⁶ (method A) or by using paired samples extracted in 0.3 ml 66 mM NaBH₄ (to reduce MTOB to MTHB) or in pre-neutralized NaBH₄ (to give the endogenous MTHB level) (method B); MTOB was estimated by difference. Method B was also used to isolate 3-dimethylsulphonio-propionaldehyde as its hydroxy derivative⁷. Metabolites were identified by their respective lability and stability in cold 2 M NaOH. ³⁵S data were corrected for recovery, determined using labelled standards, and for products formed during extraction. Protein synthesis was estimated from ³⁵S labelling of the insoluble residue; the ³⁵S was shown to be in protein-bound Met by proteolysis and TLC.

Mass spectrometry. DMSP was analysed without derivatization by FAB-MS²⁹. DMSHB was derivatized as its *t*-butyldimethylsilyl ester/ether and analysed by GC-MS with SIM after on-column nucleophile-assisted *S*-demethylation²⁹. Authentic DMSHB⁶ was used to calibrate the SIM parameters. The diagnostic fragment ion cluster at *m/z* 321 (loss of a *t*-butyl radical) was monitored at the appropriate retention time. ¹⁵N-amino acids were analysed as described¹⁴, except that amide-¹⁵N abundance was determined using *N*-ethoxycarbonyl isobutyl esters by electron impact GC-MS³⁰.

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Identification and Stereospecificity of the First Three Enzymes of 3-Dimethylsulfoniopropionate Biosynthesis in a Chlorophyte Alga¹

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Many marine algae produce 3-dimethylsulfoniopropionate (DMSP), a potent osmoprotective compound whose degradation product dimethylsulfide plays a central role in the biogeochemical S cycle. Algae are known to synthesize DMSP via the four-step pathway, L-Met → 4-methylthio-2-oxobutyrate → 4-methylthio-2-hydroxybutyrate → 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) → DMSP. Substrate-specific enzymes catalyzing the first three steps in this pathway were detected and partially characterized in cell-free extracts of the chlorophyte alga *Enteromorpha intestinalis*. The first is a 2-oxoglutarate-dependent aminotransferase, the second an NADPH-linked reductase, and the third an S-adenosylmethionine-dependent methyltransferase. Sensitive radiometric assays were developed for these enzymes, and used to show that their activities are high enough to account for the estimated in vivo flux from Met to DMSP. The activities of these enzymes in other DMSP-rich chlorophyte algae were at least as high as those in *E. intestinalis*, but were ≥20-fold lower in algae without DMSP. The reductase and methyltransferase were specific for the D-enantiomer of 4-methylthio-2-hydroxybutyrate in vitro, and both the methyltransferase step and the step(s) converting DMSHB to DMSP were shown to prefer D-enantiomers in vivo. The intermediate DMSHB was shown to act as an osmoprotectant, which indicates that the first three steps of the DMSP synthesis pathway may be sufficient to confer osmotolerance.

The tertiary sulfonium compound DMSP is synthesized and accumulated by many marine macroalgae and phytoplankton species (Blunden and Gordon, 1986; Keller et al., 1989) and by certain salt-tolerant flowering plants (Hanson and Gage, 1996). DMSP is environmentally significant because it is biodegraded to DMS, an atmospheric gas with

major roles in the global S cycle, in cloud formation, and possibly in climate regulation (Charlson et al., 1987; Malin, 1996). The DMSP produced by marine algae is the main biogenic precursor of oceanic DMS, which contributes about 1.5×10^{13} g of S to the atmosphere annually (Groene, 1995; Malin, 1996).

Like betaines, of which it is a S analog, DMSP acts as a cytoplasmic compatible solute or osmoprotectant and so has a key physiological function in adaptation to osmotic stress (Kirst, 1990; Hanson and Gage, 1996). It is also an effective cryoprotectant and contributes to the acclimation of polar algae to freezing temperatures (Karsten et al., 1996). Because DMSP has protectant properties comparable to those of betaines and does not contain N, the DMSP biosynthetic pathway is a rational target for metabolic engineering of stress resistance in N-poor, S-rich environments (Hanson and Burnet, 1994; Le Rudulier et al., 1996).

The prospect of engineering this pathway led us to investigate the steps involved in DMSP synthesis from Met in the higher plant *Wollastonia biflora* and in marine algae. The higher plant pathway proceeds via the intermediates S-methylmethionine and dimethylsulfoniopropionaldehyde (Hanson et al., 1994; James et al., 1995). The route in the marine macroalga *Enteromorpha intestinalis* and in three phytoplankton species is completely different (Gage et al., 1997) (Fig. 1). The first step in this pathway is loss of the amino group, giving the 2-oxo acid MTOB; ¹⁵N-labeling evidence strongly implies that the amino group is removed via transamination rather than deamination (Gage et al., 1997). The subsequent steps are reduction to MTHB, S-methylation to yield DMSHB, and oxidative decarboxylation to yield DMSP.

We report here the detection and partial characterization of enzymes catalyzing the first three steps of the DMSP-

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Abbreviations: AdoMet, S-adenosyl-L-Met; ammediol, 2-amino-2-methyl-1,3-propanediol; DMS, dimethylsulfide; DMSHB, 4-dimethylsulfonio-2-hydroxybutyrate; DMSP, 3-dimethylsulfoniopropionate; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MTHB, 4-methylthio-2-hydroxybutyrate; MTOB, 4-methylthio-2-oxobutyrate; MTP, 3-methylthiopropionate; TLE, thin-layer electrophoresis.

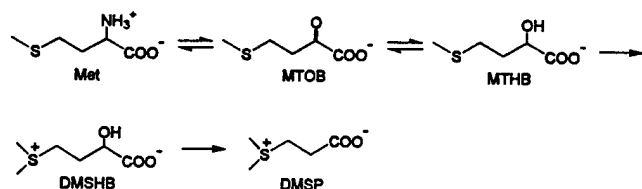


Figure 1. The biosynthesis of DMSP from Met in marine algae. In vivo isotope tracer data indicate that the first two steps are reversible (Gage et al., 1997).

synthesis pathway in the chlorophyte alga *E. intestinalis*, together with radiometric methods for their routine assay. We demonstrate that these enzymes are specific to the pathway, and that the D-enantiomers of MTHB and DMSHB are preferred. We also show that the intermediate DMSHB has osmoprotectant properties, as expected from its structure.

MATERIALS AND METHODS

The following algal species were collected from the intertidal zone or from water ≤ 2 m deep at the sites and times specified: *Enteromorpha intestinalis* (L.) Link from Marineland in Florida year-round; *Caulerpa ashmeadii* Harvey and *Udotea conglutinata* (Ellis et Solander) Lamouroux from Crystal River, FL, July, 1996; *Ulva fasciata* Delile, *Ulva reticulata* Forsskal, *Enteromorpha flexuosa* (Wulfen) J. Agardh, and *Halimeda discoidea* Decaisne from Oahu, HI, January, 1996. *E. intestinalis* for in vivo radiolabeling was used after culturing for up to 1 week, as described previously (Gage et al., 1997). For enzyme extraction, algae were frozen in liquid N_2 or dry ice within a few hours of collection and stored at -80°C ; this was shown not to affect enzyme activity. DMSP levels were estimated by a GC method (Paquet et al., 1994).

Chemicals

$[^{35}\text{S}]\text{Met}$ ($43.5 \text{ TBq mmol}^{-1}$) and $[\text{methyl-}^{14}\text{C}]\text{AdoMet}$ ($2.15 \text{ GBq mmol}^{-1}$) were purchased from NEN-DuPont and were mixed with unlabeled compounds to give the desired specific radioactivities. AdoMet was obtained from Boehringer Mannheim and was used without further purification. DL-MTHB (Ca^{2+} salt) was obtained from Fluka or Sigma; the Sigma product was recrystallized from aqueous ethanol. DMSP was purchased from Research Plus, Inc. (Bayonne, NJ). MTP was purchased from TCI (Tokyo, Japan) and neutralized with KOH. Ion-exchange resins were purchased from Bio-Rad.

Synthesis of $[^{35}\text{S}]\text{MTHB}$, $[^{35}\text{S}]\text{MTP}$, and $[^{35}\text{S}]\text{MTOB}$

D- and L- $[^{35}\text{S}]\text{MTHB}$ were prepared by incubating (24 h, 37°C) 15 MBq of $[^{35}\text{S}]\text{Met}$ [37 kBq nmol^{-1} , treated with AG-1 (formate) to remove anionic impurities] in a 750- μL reaction mixture containing 25 μmol K_2PO_4 , pH 7.4, 3 μmol GSH, 20,000 units of catalase, 2 units of L-amino acid oxidase (Sigma A-9378), 1 μmol of NADH, and 40 units of either *Staphylococcus epidermidis* D-LDH (Sigma L-9636) or

rabbit muscle L-LDH (Sigma L-2500). $[^{35}\text{S}]\text{MTP}$ was a by-product of these reactions. For the synthesis of $[^{35}\text{S}]\text{MTOB}$, the NADH and LDH were omitted and the incubation time was cut to 10 h. Products were isolated by acidifying reaction mixtures with 0.1 volume of 12 N HCl and extracting with $3 \times 3 \text{ mL}$ of ether. The combined ether extracts were back-extracted into 200 μL of 10 mM NaOH, which was concentrated in vacuo and fractionated by TLE as described below. Products were located by autoradiography, eluted with 0.5 mM β -mercaptoethanol, and stored at -80°C ; their radiochemical purity was $\geq 95\%$, as determined by TLE and TLC. The optical purity of $[^{35}\text{S}]\text{MTHB}$ enantiomers was $\geq 95\%$, as determined by susceptibility to oxidation by D- and L-LDH, as described below.

Synthesis of $[^{35}\text{S}]\text{DMSHB}$

D- and L- $[^{35}\text{S}]\text{DMSHB}$ were prepared by treating 0.93 MBq of D- or L- $[^{35}\text{S}]\text{MTHB}$ (37 kBq nmol^{-1}) with 50 μmol methanol in 0.4 mL of 6 N HCl for 4 h at 110°C (Lavine et al., 1954). $[^{35}\text{S}]\text{DMSHB}$ was isolated by ion exchange (James et al., 1995) and TLE as described below; radiochemical purity was $\geq 99\%$ as determined by TLE and TLC.

Synthesis of MTHB and DMSHB Enantiomers

Unlabeled D- and L-MTHB were synthesized from D- and L-Met by reaction with HNO_2 (Kleemann et al., 1979). Met (5 mmol) was dissolved in 4.25 mL of 0.9 M H_2SO_4 and cooled to 0°C ; 1 mL of ice-cold 6.2 M NaNO_2 was added dropwise, and the reaction mixture was then incubated for 2 h at 22°C . The MTHB product was extracted into $4 \times 3 \text{ mL}$ of ether, dried in a stream of N_2 , and dissolved in 1 mL of water. MTHB was then purified by passage onto a 1.25-mL AG-1 (OH^-) column, from which it was eluted with 6 mL of 2.5 N HCl, extracted again with ether, and lyophilized. Purity was about 98%, as determined by TLC and TLE. D- and L-DMSHB were prepared from 0.12 mmol of the corresponding form of MTHB by heating at 110°C for 2 h with 0.25 mmol of methanol in 0.5 mL of 6 N HCl. After removing the HCl in vacuo, DMSHB was isolated by ion exchange (James et al., 1995). The product was lyophilized and freed of a small amount (10%) of putative dimer by hydrolysis with 0.1 N HCl at 100°C for 2 h. The optical purity of the D and L forms of MTHB and DMSHB was estimated as $\geq 92\%$ by circular dichroism measurements. DL-DMSHB was synthesized from DL-MTHB by the method of Toennies and Kolb (1945).

TLE and TLC

TLE separations were on glass-backed 0.1-mm cellulose plates (Merck, Darmstadt, Germany) at 1.8 kV for 20 min at 4°C . The buffers were pyridine:glacial acetic acid:water (1:1:38, v/v/v) for thioethers, and 1.5 N formic acid for sulfonium compounds. TLC of thioethers and amino acids was carried out on cellulose plates developed with *n*-butanol:glacial acetic acid:water (60:20:20, v/v/v); sulfonium compounds were separated on plastic-backed 0.25-mm silica gel G plates (Machery-Nagel, Düren, Ger-

many) developed with methanol:acetone:concentrated HCl (90:10:4, v/v/v). Compounds were visualized using the spray reagents described previously (James et al., 1995).

Enzyme Extraction

Tissue was pulverized in liquid N₂ and extracted with 3.5 volumes of buffer A (50 mM Bis-Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane]-HCl, pH 8.0, 5 mM DTT, 2 mM K₂EDTA, and 1 mg mL⁻¹ BSA); for MTHB S-methyltransferase in *E. intestinalis*, the pH was 7.0 and BSA was omitted. Subsequent steps were performed at 4°C. The brei was centrifuged at 10,000g for 10 min, and the supernatant was desalted on Sephadex G-25 equilibrated in buffer A. For Met aminotransferase and MTOB reductase assays the supernatant was then concentrated 10-fold with a Centricon-30 (Amicon). For all three DMSP synthesis enzymes, the desalted supernatants contained ≥70% of the activity present in the brei. Centrifugation at 100,000g for 1 h did not pellet activity of any of the enzymes. Desalted supernatants were in some cases flash-frozen in liquid N₂ and stored at -80°C; this did not affect enzyme activity. Enzyme assays were carried out under conditions in which product formation was linear with respect to time and enzyme concentration.

Enzyme Assays

MDH, Asp Aminotransferase, and Ala Aminotransferase

MDH assays contained 0.1 M Tris-acetate, pH 8.0, 0.2 mM NADH, and 2.5 mM oxaloacetate; oxaloacetate-dependent oxidation of NADH was followed by the fall in A₃₄₀. Asp aminotransferase assays contained 0.1 M potassium phosphate buffer, pH 7.5, 25 mM L-Asp, 5 mM 2-oxoglutarate, 0.2 mM NADH, and 60 units mL⁻¹ of porcine heart MDH (Sigma M-2634); 2-oxoglutarate-dependent NADH oxidation was monitored. Ala aminotransferase was assayed in the same way except that 100 mM L-Ala replaced Asp, and 120 units mL⁻¹ rabbit muscle LDH (Sigma L-2500) was used in the coupling reaction.

Met Aminotransferase and Met Oxidase

Standard aminotransferase assays (60 μL final volume) contained 0.1 M ammonium-HCl, pH 9.1, 100 μM L-Met (7–22 kBq, treated with AG-1 [formate]) and 1 mM 2-oxoglutarate. Met oxidase assays were the same except that 2-oxo acid was excluded. After 1 h at 25°C, reactions were stopped on ice and acidified with 10 μL of 2.5 N HCl after adding carrier Met and MTOB (0.2 μmol). The [³⁵S]MTOB formed was extracted into 0.8 mL of ether, then back-extracted into 100 μL of 10 mM NaOH, of which 80 μL was taken for scintillation counting. Aminotransferase activity with other 2-oxo acids was assayed using a [³⁵S]Met concentration of 25 μM. Aminotransferase and oxidase data were corrected for MTOB recovery; aminotransferase data were corrected for Met oxidase activity. TLC and TLE confirmed that the product of aminotransferase and oxidase reactions was [³⁵S]MTOB. Aminotransferase activity

was assayed in the Met-synthesis direction in 30-μL reaction mixtures containing 0.1 M ammonium-HCl, pH 9.1, 3.1 μM [³⁵S]MTOB (0.63 kBq), and 10 mM amino acid. Incubation and carriers were as above. The [³⁵S]Met product was isolated by passage onto 1-mL AG-50 (H⁺) columns, washing with 25 mL of water, and eluting with 5 mL of 2.5 N HCl. The product was shown to be [³⁵S]Met by TLC. Data were corrected for Met recovery and for ³⁵S found in the AG-50 eluate of blank assays lacking amino acid.

MTOB Reductase

Standard assays (final volume 30 μL) contained 0.1 M ammonium-HCl, pH 8.0, 150 μM NADPH, and 30 μM [³⁵S]MTOB (7.4 kBq). After incubation for 1 h at 25°C, 50 nmol MTOB and 100 nmol MTHB carriers were added, followed at once by 50 μL of 10 mM 2,4-dinitrophenylhydrazine in 2.5 N HCl. The samples were then incubated for 1 h at 22°C and extracted with 0.5 mL of ether. The ether phase was back-extracted with 50 μL of 10 mM NaOH containing 3 μL of 1 M acetic acid, which was concentrated in vacuo and subjected to TLC. [³⁵S]MTHB zones were located with iodoplatinate reagent and quantified by scintillation counting. Data were corrected for MTHB recovery and for ³⁵S in the MTHB zone in assays without NADPH. Assays of MTHB oxidation (final volume 50 μL) contained 0.1 M ammonium-HCl, pH 8.0, 30 μM D-[³⁵S]MTHB (46 kBq), and 1 mM NADP. Incubation was for 2 h at 25°C. Oxidation products were measured as described below for determination of [³⁵S]MTHB configuration.

D-MTHB S-Methyltransferase

Standard assays (final volume 50 μL) contained 50 mM Bis-Tris-HCl, pH 7.0, 1 mM AdoMet, and 25 μM D-[³⁵S]MTHB (1.9–3.7 kBq). After incubation at 22°C for 1 h, 1 mL of 0.2 M DMSHB carrier was added and the mixture was immediately applied to a 1-mL mixed-resin column (AG-1 [OH⁻]:BioRex 70 [H⁺], 2:1, v/v, firmly packed). The [³⁵S]DMSHB product was eluted with 5 mL of water and quantified by scintillation counting. In some cases the enzyme was assayed using unlabeled MTHB and 100 μM [methyl-¹⁴C]AdoMet (3.7 kBq). These reactions were stopped after 1 h by adding 25 μL of 10% (w/v) TCA, 2 μL of 100 mM DMSHB, and 215 μL of an activated charcoal suspension (38 mg mL⁻¹) in 0.1 N acetic acid to bind AdoMet (Cook and Wagner, 1984). After centrifuging for 5 min at 14,000g, [¹⁴C]DMSHB in the supernatant was quantified by scintillation counting. Values were corrected for DMSHB recovery and for blanks lacking the unlabeled substrate. The identities of the ³⁵S- and ¹⁴C-labeled DMSHB reaction products were confirmed by TLE and TLC.

Configuration of MTHB

[³⁵S]MTHB was prepared from [³⁵S]MTOB by scaling up the standard MTOB reductase assay, and then purified by TLE. Samples (2.6 kBq, 110 pmol) were incubated for 20 h in darkness at 30°C in 30-μL reaction mixtures containing 50 mM ammonium-HCl, pH 9.0, 5 mM NAD⁺, either 6 units

of *S. epidermidis* D-LDH (Sigma L-9636) or 3 units of rabbit muscle L-LDH (Sigma L-1254), and 0.3 unit of *Photobacterium fischeri* NAD(P)H:FMN oxidoreductase. Controls of authentic D- and L-[^{35}S]MTHB were treated the same way. The reactions were stopped with 70 μL of 0.54 N HCl, mixed with carrier MTHB, MTOB, and MTP (100 nmol each), and extracted with 3×0.8 mL of ether. The combined ether fraction was back-extracted into 70 μL of 10 mM NaOH, which was concentrated in vacuo and subjected to TLE. [^{35}S]MTHB, [^{35}S]MTOB, and [^{35}S]MTP (a breakdown product of [^{35}S]MTOB) were located by autoradiography and by I_2 staining, and quantified by scintillation counting. Control reactions without LDH showed no [^{35}S]MTHB oxidation.

In Vivo Radiotracer Experiments

Samples (100 mg fresh weight) of tissue cut from the basal 1- to 2-cm region of *E. intestinalis* fronds were incubated in 0.5 mL of 0.2- μm filtered seawater containing 37 or 74 kBq (1 or 2 nmol) of [^{35}S]MTHB or [^{35}S]DMSHB. For experiments with [^{35}S]MTHB, the pH was lowered to an initial value of 5.7 by adding Mes-KOH (final concentration 100 mM) to the seawater. Incubation was at 22°C in the light, as described previously (Gage et al., 1997); label uptake was monitored by sampling the medium. After incubation, the tissue was rinsed for 5 min in seawater, blotted dry, and extracted using a methanol-chloroform-water procedure (Hanson et al., 1994; James et al., 1995). Water-soluble metabolites were fractionated by ion-exchange chromatography and analyzed by TLC and TLE. Incorporation of ^{35}S into the insoluble fraction was estimated by scintillation counting after suspending samples in Ready Gel (Beckman) containing 50% (v/v) water; the counting efficiency of this system was determined to be 65%.

Bacterial Osmoprotection Experiments

The *Escherichia coli* strains used were K-10 and FF4169, an *otsA* (trehalose-deficient) mutant of K-12 (Gjæver et al., 1988). Experiments with K-10 were as described by Hanson et al. (1991), except that the medium was that of Neidhardt et al. (1974) and cultures were inoculated with cells growing exponentially in the presence of NaCl. FF4169 was grown at 37°C to stationary phase in M63 medium (Miller, 1972), pH 7.0, containing 0.2% (w/v) Glc. This culture was used to inoculate experimental M63 media (25 mL) containing NaCl and various supplements (final concentration 1 mM). Supplement solutions were adjusted to pH 7.0 and filter-sterilized before addition; care was taken to avoid alkaline pH while neutralizing the DMSP solution. Experimental cultures were incubated at 37°C, and growth was monitored by the change in A_{600} .

RESULTS

Estimation of the Rate of DMSP Synthesis in Vivo

To provide a benchmark for the activities of DMSP pathway enzymes in *E. intestinalis*, we first estimated the in

vivo flux of Met to DMSP by computer modeling of published [^{35}S]Met-tracer kinetic data for this alga (Gage et al., 1997). The computer model used was that described by Mayer et al. (1990). The starting free pool size of free Met was taken to be up to 10 nmol g^{-1} fresh weight, based on published values for the chlorophyte alga *Chlorella sorokiniana* (Giovannelli et al., 1980). The flux values from Met to DMSP that gave satisfactory fits to the published labeling patterns of MTHB, DMSHB, and DMSP ranged from 1.2 to 4.2 nmol $\text{h}^{-1} \text{g}^{-1}$ fresh weight.

Met Aminotransferase Activity

Met aminotransferase activity was readily detected in assays containing MTOB or 2-oxoglutarate as the amino acceptor for [^{35}S]Met, but the activities found with other 2-oxo acids were far lower (Table I). 2-Oxoglutarate was therefore used to further characterize the activity. Activity was not inhibited by a 5-fold excess of unlabeled D-Met, indicating that it was not due to the tandem action of a racemase and a D-Met aminotransferase. The pH profile showed an optimum at 9.1, and 40 to 60% of optimal activity in the physiological range of 7.5 to 8.0. The affinity for Met was high, as shown by velocity versus Met concentration curves (Fig. 2A). Double-reciprocal plots indicated that half-maximal velocity was reached at 30 μM Met, although they also suggested the presence of a minor activity with much lower affinity (not shown). The 2-oxoglutarate concentration giving half-maximal velocity was 400 μM at 100 μM [Met].

Table I. Substrate preference of Met aminotransferase activity

2-Oxo acid preference was determined in reactions containing desalted extract equivalent to 5.5 mg fresh weight, 25 μM L-[^{35}S]Met, and an optimal concentration of 2-oxo acid (0.1 mM for MTOB and 1 mM for all others). L-Amino acid preference was determined in reactions containing extract equivalent to 0.6 to 5.5 mg fresh weight, 3.1 μM [^{35}S]MTOB, and 10 mM amino acid. Data for 2-oxo acids and amino acids were obtained at pH 9.1 and are expressed relative to the activities obtained with MTOB (21.7 pkat g^{-1} fresh weight) and Met (13.0 pkat g^{-1} fresh weight), respectively.

Substrate	Relative Activity
	%
2-Oxo acids	
MTOB	100
2-Oxoglutarate	81.0
Phenylpyruvate	14.1
Pyruvate	9.7
Oxaloacetate	9.1
Glyoxylate	2.8
Hydroxypyruvate	<1.0
L-Amino acids	
Met	100
Glu	94.9
Phe	6.0
Ala	5.3
Asp	1.3
Gly	<0.2
Ser	<0.2
Gln	8.5
Asn	1.2

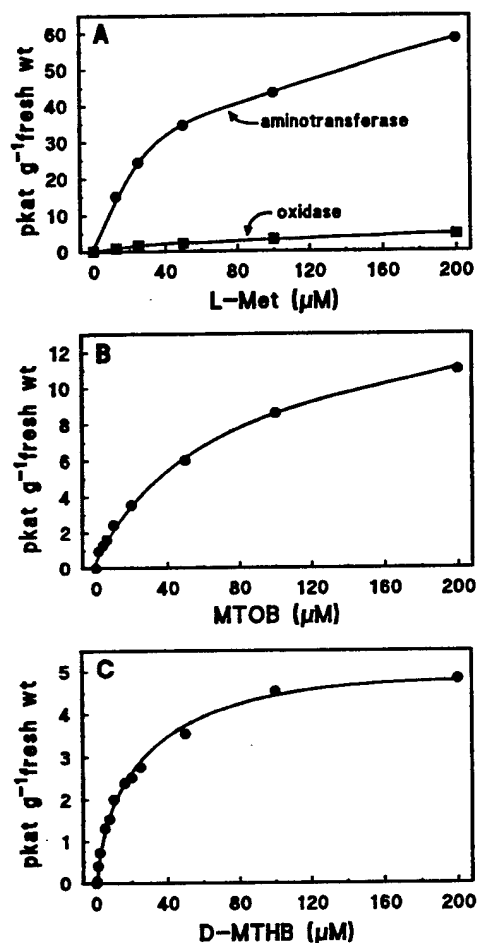


Figure 2. Plots of velocity versus substrate concentration for enzyme activities implicated in DMSP biosynthesis. Activities were measured using desalted *E. intestinalis* extract equivalent to 5 to 6.5 mg fresh weight per assay. A, Met aminotransferase, assayed in the presence of 1 mM 2-oxoglutarate. Met oxidase was assayed in the absence of 2-oxo acid. B, MTOB reductase, assayed using 200 μ M NADPH. C, D-MTHB 5-methyltransferase, assayed using 1 mM AdoMet. Experiments with each activity were done at least twice, with similar results to those shown. The L-Met and D-MTHB concentrations giving half-maximal velocity given in the text were estimated from double-reciprocal plots.

Consistent with the strong preference for 2-oxoglutarate as the amino acceptor, assays in the reverse (Met synthesis) direction using [35 S]MTOB showed that L-Glu was by far the best amino donor after Met itself (Table I). All other amino acids tested were ≥ 10 -fold less effective, including Gln and Asn. Met aminotransferase activity was not increased by including pyridoxal-5'-phosphate (0.1 mM) in extraction and assay buffers. This is not unusual, since the pyridoxal-5'-phosphate of plant aminotransferases is typically tightly bound and few are activated by its addition (Ireland and Joy, 1985).

Met Oxidase Activity

E. intestinalis extracts catalyzed the slow conversion of [35 S]Met to [35 S]MTOB in the absence of 2-oxo acid (Fig.

2A). This activity was ascribed to a nonspecific L-amino acid oxidase because it was decreased by lowering the O_2 concentration and increased by raising it, and because it was strongly suppressed when unlabeled amino acids that are good substrates for other L-amino acid oxidases (Meister, 1965) were present (Table II). Figure 2A shows that oxidase activity was only 8% of aminotransferase activity even at a Met concentration of 200 μ M. Since cytoplasmic levels of Met in algae and other plants are most probably ≤ 200 μ M (Giovanelli et al., 1980), the oxidase seemed unlikely to mediate much MTOB synthesis in vivo and was not investigated further.

MTOB Reductase Activity

E. intestinalis extracts showed NADPH- and NADH-dependent MTOB reductase activities of similar magnitude at saturating NAD(P)H levels, but half-maximal rates were attained at 30 μ M NADPH versus 650 μ M NADH (Fig. 3). The two activities were not additive, consistent with their being due to the same enzyme(s) (Fig. 3B). Because NAD(P)H levels in plant cytoplasm are typically not more than 50 to 150 μ M (Heber and Santarius, 1965; Hampp et al., 1984), the NADPH-linked activity appeared likely to be the dominant one in vivo. This activity was therefore characterized further. It was highest at pH 8.0, and about 70% as high at pH 7.0. Velocity versus MTOB concentration plots showed clearly that affinity for MTOB was high (Fig. 2B). Since double-reciprocal plots were nonlinear (showing apparent negative cooperativity), the MTOB concentration giving half-maximal velocity could not be determined precisely, but appeared to be approximately 40 μ M. LDH and acetohydroxy acid isomeroreductase can both catalyze the reduction of various 2-oxo acids, and LDH is specifically known to attack MTHB (Meister, 1957; Umbarger, 1996). However, MTOB reductase activity was not attributable to either of these enzymes since [35 S]MTOB reduction was scarcely inhibited ($\leq 30\%$) by a 200-fold excess of unlabeled pyruvate or 2-oxoisovalerate (data not shown).

The configuration of the [35 S]MTHB produced by the *E. intestinalis* MTOB reductase was determined by testing it as a substrate for purified D- and L-LDH; authentic D- and L-[35 S]MTHB were included as controls (Table III). The controls behaved as predicted, and the reductase product

Table II. Characteristics of 2-oxo acid-independent Met oxidation

Desalted extract (equivalent to 17 mg fresh weight) was incubated with 100 μ M L-[35 S]Met for 1 h in 60- μ L reaction mixtures in 1-mL tubes. For O_2 and N_2 treatments, the tubes were closed with serum caps and purged for 2 min before injecting L-[35 S]Met to start the reaction. For other treatments the tubes contained air. Values in parentheses are relative to the control (=100).

Treatment	Met Oxidation pkat g ⁻¹ fresh wt
Control (no additions)	1.69 (100)
N ₂ -Purged	0.57 (34)
O ₂ -Purged	2.98 (176)
+5 mM L-Phe	0.15 (9)
+5 mM L-Leu	0.17 (10)

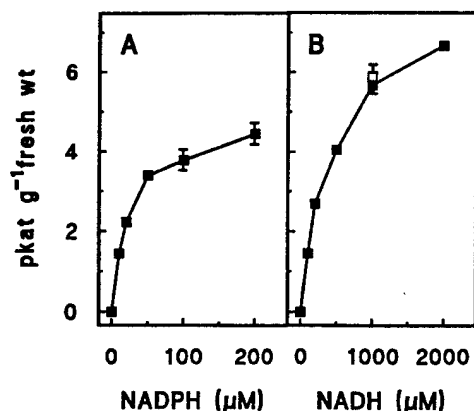


Figure 3. NADPH-dependent (A) and NADH-dependent (B) MTOB reductase activity. Reaction mixtures contained desalted *E. intestinalis* extract equivalent to 8.6 mg fresh weight, 30 μM [^{35}S]MTOB, and various concentrations of NADPH or NADH. Data points are means \pm SE ($n = 3$). Where error bars are not shown they were smaller than the symbols. The open symbol in B shows the activity given by 1 mM NADH plus 100 μM NADPH.

was attacked only by D-LDH. The in vitro product of *E. intestinalis* MTOB reductase is therefore D-MTHB; it is also very probable that the D-enantiomer predominates in vivo, as shown below. Knowing that reductase produces D-MTHB, it was of interest to seek the reverse reaction, i.e. NADP-dependent D-[^{35}S]MTHB oxidation. This was readily detectable; in the presence of 35 μM D-[^{35}S]MTHB and 1 mM NADP at pH 8.0, the reaction rate was 0.4% of the forward rate in comparable conditions. Adding NAD(P)H:FMN oxidoreductase plus FMN (to remove NADPH) did not accelerate the reverse reaction.

MTHB S-Methyltransferase Activity

Consistent with the product of MTOB reductase being the D form of MTHB, *E. intestinalis* extracts catalyzed

Table III. Evidence that the product of MTOB reductase is the D-enantiomer of MTHB

[^{35}S]MTHB synthesized from [^{35}S]MTOB using desalted *E. intestinalis* extract was incubated with *S. epidermidis* D-LDH or rabbit muscle L-LDH. As controls, comparable amounts of authentic D- and L-[^{35}S]MTHB were treated in the same way. Reaction mixtures were fractionated by TLE, and radioactivity in MTHB, MTOB, and MTP was quantified. MTP is formed from MTOB by spontaneous decarboxylation (Gage et al., 1997). [^{35}S]MTHB oxidation was therefore expressed as the percentage of ^{35}S recovered in MTOB and MTP. Corrections were applied for traces ($\leq 1\%$) of these compounds present in the [^{35}S]MTHB substrates.

Substrate	Specificity of LDH Used	[^{35}S]MTHB Oxidation
		%
Reductase product	D	15.5
	L	<0.5
D-[^{35}S]MTHB	D	16.0
	L	<0.5
L-[^{35}S]MTHB	D	<0.5
	L	14.0

AdoMet-dependent S-methylation of D-MTHB but not L-MTHB. This was demonstrated in two types of assays based on enzymatically synthesized D- and L-[^{35}S]MTHB or on chemically synthesized, unlabeled D- and L-MTHB as substrates (Table IV). There was no detectable activity with MTP as substrate (Table IV), which is in accord with the in vivo radiotracer evidence against a role for this compound in DMSP synthesis in *E. intestinalis* (Gage et al., 1997). Nor did the enzyme attack other naturally occurring thioethers (3-methylthiopropylamine, D- or L-Met, L-S-methylcysteine), as judged from their complete failure to inhibit D-[^{35}S]MTHB methylation when present in unlabeled form in 40-fold excess (data not shown). The D-MTHB methyltransferase activity had a broad pH optimum in the region of 6.5 to 8.0. Plots of velocity versus [D-MTHB] showed that activity was half-maximal at 8 μM D-MTHB (Fig. 2C); the AdoMet concentration giving half-maximal velocity was 30 μM at 25 μM [D-MTHB].

Comparative Biochemistry of DMSP-Accumulating and Nonaccumulating Algae

Evidence that the Met aminotransferase, MTOB reductase, and D-MTHB methyltransferase activities found in *E. intestinalis* are specific to DMSP synthesis was sought by assaying these enzymes in extracts of six other marine chlorophyte algae, three with DMSP and three without (Table V). The activities of three housekeeping enzymes (Asp and Ala aminotransferases and MDH) were also measured as a check on the quality of the extracts. All six algae had housekeeping enzyme activities comparable to those of *E. intestinalis* (Table V). Those that accumulated DMSP all showed Met aminotransferase, MTOB reductase, and D-MTHB methyltransferase activities at least as high as those of *E. intestinalis*. In contrast, the algae lacking DMSP had about 30-fold less Met aminotransferase activity than *E. intestinalis*, ≥ 20 -fold less MTOB reductase, and no detectable methyltransferase. All six algae had very low levels of Met oxidase (0.5–2.3 pkat g^{-1} fresh weight; not shown), providing further evidence that this activity is not importantly related to DMSP synthesis.

Table IV. Enantiomer and substrate specificity of S-methyltransferase activity

Desalted extract equivalent to 3.8 mg fresh weight was incubated with 25 μM [^{35}S]MTHB or [^{35}S]MTP and 1 mM AdoMet, or with 25 μM unlabeled MTHB and 0.1 mM [methyl- ^{14}C]AdoMet. Incorporation of label into methylated product (DMSHB or DMSP) was measured. The L-[^{35}S]MTHB and L-MTHB could have contained small amounts of the D forms (see "Materials and Methods"), which may account for the slight activity observed with the L enantiomers.

Substrate	Enantiomer	Methyltransferase Activity
		pkat g^{-1} fresh wt
[^{35}S]MTHB	D	2.42
	L	0.03
Unlabeled MTHB	D	2.44
	L	0.11
[^{35}S]MTP	–	<0.02

Table V. Activities of putative enzymes of DMSP synthesis in various marine chlorophyte algae

Activities were measured in *E. intestinalis*, in three other algal species that accumulate DMSP, and in three that do not. Desalted extracts were prepared as described under "Materials and Methods" for *E. intestinalis*. Asp and Ala aminotransferases (Asp-AT and Ala-AT) and MDH were assayed spectrophotometrically, and Met aminotransferase (Met-AT), MTOB reductase (MTOB-R), and D-MTHB methyltransferase (MTHB-MT) were assayed radiochemically, as described in "Materials and Methods."

Species	DMSP Content $\mu\text{mol g}^{-1}$ fresh wt	Housekeeping Enzyme Activity			DMSP Pathway Enzyme Activity		
		MDH	Asp-AT	Ala-AT	Met-AT	MTOB-R	MTHB-MT
		nkat g^{-1} fresh wt	nkat g^{-1} fresh wt	nkat g^{-1} fresh wt	pkat g^{-1} fresh wt	pkat g^{-1} fresh wt	pkat g^{-1} fresh wt
<i>E. intestinalis</i>	19.4	30	4.5	3.33	30.3	3.71	3.44
<i>E. flexuosa</i>	17.5	155	18.0	4.55	87.2	4.08	13.0
<i>U. reticulata</i>	18.5	138	12.4	1.25	29.7	4.44	7.39
<i>U. fasciata</i>	12.5	185	7.9	2.98	35.3	5.22	7.94
<i>H. discoidea</i>	<0.03	71	22.5	0.63	0.9	<0.10	<0.02
<i>C. ashmeadii</i>	<0.03	116	19.7	2.30	1.3	0.20	<0.02
<i>U. conglutinata</i>	<0.03	22	15.0	1.08	1.0	0.14	<0.02

Confirmation of D-Enantiomer Preferences in Vivo

E. intestinalis fronds converted D-[^{35}S]MTHB to DMSP very efficiently (Table VI). As reported by Gage et al. (1997), the L form was also converted to DMSP, but 5-fold less efficiently than the D form. These data confirm the preference of DMSP synthesis for D-MTHB in vivo. Much of the L-MTHB was metabolized to protein-bound Met, and more label from the L form remained in the intermediate DMSHB (Table VI), presumably because the ^{35}S flux was small in relation to the pool size of DMSHB. The conversion of L-MTHB to Met indicates that it can be oxidized to MTOB and then transaminated to yield Met, as occurs in other organisms (Livesey, 1984; Miyazaki and Yang, 1987). MTOB formed in this way could also be converted to D-MTHB, which would explain the small ^{35}S flux from L-MTHB to DMSHB and DMSP.

Since the MTHB methyltransferase produces D-DMSHB, the enzyme(s) converting DMSHB to DMSP might be expected to prefer D-DMSHB. Supplying D- and L-[^{35}S]DMSHB confirmed this expectation; the D form was converted to DMSP 9-fold more efficiently than the L form (Table VI).

Table VI. Metabolism of enantiomers of MTHB and DMSHB by *E. intestinalis*

Algal fronds (0.1 g fresh weight) were incubated with 74 kBq (2 nmol) of [^{35}S]MTHB or 37 kBq (1 nmol) of [^{35}S]DMSHB in 0.5 mL of seawater for 6 h in the light. For [^{35}S]MTHB, the initial pH of the seawater was adjusted to 5.7 with Mes-KOH. Precursor uptake was estimated from disappearance from the medium. Radiolabel incorporated into the insoluble fraction was shown to be in protein-bound Met by protease digestion and acid hydrolysis (Gage et al., 1997). The data are representative of three separate experiments.

^{35}S -Precursor	Precursor Uptake kBq	^{35}S Distribution		
		Insoluble	DMSHB	DMSP
		% of uptake		
D-MTHB	9.1	5.1	<0.1	77.2
L-MTHB	8.0	16.4	27.4	16.1
D-DMSHB	29.3	6.9	23.7	69.5
L-DMSHB	34.8	3.6	71.7	8.1

The modest conversion of the L form to DMSP is in accord with our previous data (Gage et al., 1997).

Osmoprotection of *E. coli* by DMSHB

E. intestinalis does not accumulate high levels of the intermediate DMSHB (Gage et al., 1997). However, from its structure this compound is predicted to be an effective osmoprotectant, whereas its precursor, MTHB, is not (Yancey, 1994). This prediction was tested using standard osmoprotection bioassays with two *E. coli* strains; DMSHB and MTHB were compared with DMSP and Glycine betaine as benchmarks. Figure 4 shows data for the osmosensitive strain FF4169; results with strain K-10 were similar except for a higher growth rate in the absence of osmoprotectants. Although less effective than Glycine betaine and DMSP, DL-

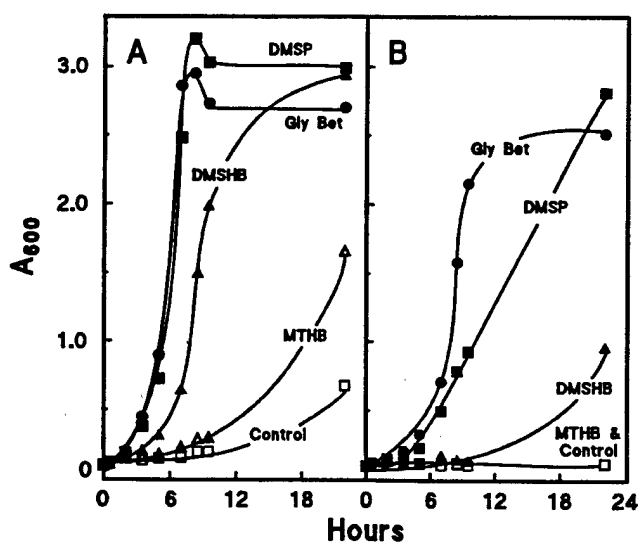


Figure 4. Osmoprotection by DMSHB. The osmosensitive (trehalose-deficient) *E. coli* strain FF4169 was grown aerobically at 37°C in M63-Glc medium containing 0.45 M (A) or 0.65 M (B) NaCl alone (control) and with 1 mM DL-DMSHB, DL-MTHB, DMSP, or Glycine betaine (Gly Bet). Growth was monitored by A_{600} .

DMSHB was much more potent than DL-MTHB, which protected the cells slightly or not at all. Tested separately, the D and L forms of DMSHB and MTHB behaved like the racemic mixtures. The osmoprotective effect of DMSHB involved its intracellular accumulation, but not any metabolism. Thus, when 1 mM D- or L-[³⁵S]DMSHB was included in media containing 0.5 M NaCl, all of the label taken up by K-10 cells was shown by TLE to be in DMSHB; the intracellular concentration of [³⁵S]DMSHB was estimated to be 0.5 to 0.7 M by assuming cell volume to be 0.4 fL (Hanson et al., 1991).

DISCUSSION

In the marine alga *E. intestinalis*, the first three steps of the DMSP biosynthesis pathway are Met → MTOB → MTHB → DMSHB (Fig. 1). We report here enzyme activities that catalyze these steps, and show that the D-enantiomers of the chiral intermediates MTHB and DMSHB are very strongly preferred. As summarized in Table VII, all three enzymes show high affinities for their substrates and have extractable activities 4- to 25-fold above our highest estimate of the in vivo rate of DMSP synthesis (4.2 nmol h⁻¹ g⁻¹ fresh weight). Moreover, much of the activity was probably not extracted because our extracts contained about 0.5 mg protein g⁻¹ fresh weight, whereas the total protein content (Kjeldahl N × 6.25) of *E. intestinalis* fronds was found to be 5.9 mg g⁻¹ fresh weight, in accord with published values (2–10 mg g⁻¹ fresh weight; Edwards et al., 1988).

The conversion of MTOB to Met via transamination is the last step in the Met salvage pathway whereby the methylthio moiety of 5'-methylthioadenosine is recycled to Met (Cooper, 1996). Since this pathway is ubiquitous, all algae would be expected to show some Met aminotransferase activity, and indeed this was the case (Table V). However, the activity was 30- to 100-fold higher in the DMSP-accumulating algae, indicating that DMSP synthesis involves either overexpression of a housekeeping Met aminotransferase or expression of a novel enzyme. A novel enzyme appears more likely for two reasons.

First, the strong preference for Glu as an amino donor distinguishes the *E. intestinalis* activity from the aminotransferases known to be involved in Met salvage in plants, animals, and bacteria, which appear to prefer Gln and Asn (Cooper, 1996). Second, the estimated apparent K_m for Met is exceptionally low (30 μ M); aminotransferases typically have K_m values for amino acids in the mM range (Jenkins and Fonda, 1985), and Gln and Asn aminotransferases are no exception (Cooper and Meister, 1985). The *E. intestinalis* enzyme is also clearly distinct from a Met-glyoxylate aminotransferase involved in glucosinolate synthesis in crucifers, which shows little activity with 2-oxoglutarate as amino acceptor (Chapple et al., 1990), and from a nonspecific, low-affinity Met aminotransferase in pea (Kutacek, 1985). Because DMSP represents about 90% of the reduced S in *E. intestinalis*, a high-affinity aminotransferase may be needed to sustain a high Met flux to DMSP in the face of competition for Met from AdoMet and methionyl-tRNA synthetases, which can have high affinities for Met (Burbaum and Schimmel, 1992; Schröder et al., 1997).

As with Met transamination, a small capacity to convert MTOB to MTHB may be widespread, because MTHB (of undetermined configuration) has been detected as a metabolite of radiotracer Met or MTOB in diverse higher and lower plants, including algae that lack DMSP (Pokorny et al., 1970; Kushad et al., 1983; Miyazaki and Yang, 1987). However, MTHB formation in these cases may be just a reversible side reaction of the Met salvage pathway (Miyazaki and Yang, 1987), which would be expected to carry very little flux compared with DMSP synthesis. Consistent with this interpretation, DMSP-free algae had no more than 5% of the MTOB reductase activity found in DMSP accumulators.

Unlike the Met → MTOB → MTHB reaction sequence, the conversion of MTHB to DMSHB is known only in association with DMSP synthesis, for which it may be the committing step (Gage et al., 1997). The D-MTHB S-methyltransferase catalyzing this step would thus appear to be a novel enzyme of potential regulatory importance. Our finding that its product is an osmoprotectant has both evolutionary and metabolic engineering implications. Supposing that, like many extant plants, ancient algae had some capacity to form MTHB, evolution of an MTHB methyltransferase could have enabled some DMSHB synthesis to occur and so conferred a selective advantage. DMSHB might thus have served as an ancestral sulfonium osmoprotectant from which synthesis of the more potent protectant DMSP later evolved. In this connection it is noteworthy that two other algal sulfonium compounds, gonyauline (*cis*-2-[dimethylsulfonio]cyclopropanecarboxyl-ate) (Nakamura et al., 1992) and 4-dimethylsulfonio-2-methoxybutyrate (Blundell and Gordon, 1986), are structurally related to DMSHB and that both could hypothetically be derived from it by single-step reactions.

With respect to metabolic engineering of the DMSP pathway in crops or other organisms, it may be necessary to get only as far as DMSHB to achieve a useful degree of osmotic stress resistance. Whereas genes for Met aminotransferase, MTOB reductase, and MTHB methyltransferase might all be required for this, some crucifers have high Met-glyoxylate aminotransferase activities (4–50 pkat g⁻¹ fresh

Table VII. Estimated activities and affinities of DMSP-synthesis enzymes

Approximate maximal activity (V_{max}) values are means \pm SE of three to five experiments with different batches of *E. intestinalis*, and are given in units of nmol h⁻¹ g⁻¹ fresh weight for comparison with in vivo flux estimates. Each enzyme was assayed at its pH optimum. The substrate concentrations giving half-maximal velocity ($S_{0.5}$) were calculated from the data shown in Figure 2 and may be taken as rough estimates of apparent K_m values.

Enzyme	Enzyme Characteristics	
	V_{max}	$S_{0.5}$
	nmol h ⁻¹ g ⁻¹	μ M
Met aminotransferase	103 \pm 21	30
MTOB reductase	30 \pm 4	40 ^a
D-MTHB methyltransferase	19 \pm 2	8

^a Approximate value only (see text).

weight; Glover et al., 1988; Chapple et al., 1990) and in such cases just two engineered genes would perhaps suffice.

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CHAPTER 10

SALINITY, OSMOLYTES AND COMPATIBLE SOLUTES

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Abstract

In order to maintain low cytosolic Na⁺, and cytosolic K⁺ concentrations within narrow limits (100 - 150 mM) across a broad range of external and vacuolar concentrations of NaCl, it is essential that other solutes be accumulated in the cytoplasm to keep this compartment in osmotic balance with the external medium and vacuole. Such solutes should be osmolytes that are non-toxic and "compatible" with cytoplasmic enzymes over wide concentration ranges. A number of such solutes have been identified, their pathways of synthesis elucidated, and progress has been in isolating genes encoding key enzymes of their biosynthetic pathways.

10.1. Introduction

In general, the enzymes of salt-tolerant plants are no more tolerant of deleterious inorganic ions, such as Na⁺, than are the enzymes of salt-sensitive plants (Flowers *et al.*, 1977). Plant adaptation to saline environments therefore requires the maintenance of low levels of Na⁺ in the cytoplasm, achieved in part by accumulation of Na⁺ in the vacuole (Flowers *et al.*, 1977). Eukaryotic cells are remarkably uniform in maintaining a fairly constant cytoplasmic K⁺ concentration of between 100 and 150 mM (Wyn Jones *et al.*, 1977). In contrast to Na⁺, K⁺ is essential for many processes in plants, including protein synthesis, membrane transport processes, generation of turgor, charge balance, and activation of certain enzymes (Leigh and Wyn Jones, 1984; Matoh *et al.*, 1988). The primary role of K⁺ in the cytoplasm is for protein synthesis to maintain the association between tRNA and ribosomes during translation (Leigh and Wyn Jones,

1984). However, concentrations of K^+ exceeding 180 mM are inhibitory to protein synthesis because such concentrations promote dissociation of polysomes and conformational changes in monosomes (Brady *et al.*, 1984). In general, K^+ is less inhibitory than Na^+ to enzyme function, but nevertheless both ions can be regarded as "strongly perturbing" to macromolecular structure and function at concentrations above 200 mM (Hochachka and Somero, 1984; Yancey, 1994). The sensitivity of protein synthesis to inorganic ions may set the boundaries for cell ionic strength (Leigh and Wyn Jones, 1984; Yancey, 1994).

Given the need to maintain low cytosolic Na^+ , and cytosolic K^+ concentrations within narrow limits (100 - 150 mM) across a broad range of external and vacuolar concentrations of NaCl, it is essential that other solutes be accumulated in the cytoplasm to keep this compartment in osmotic balance with the external medium and vacuole. Ideally, such solutes should be osmolytes that are non-toxic and "compatible" with cytoplasmic enzymes over wide concentration ranges (Wyn Jones *et al.*, 1977).

10.2. Compatible and non-compatible solutes

Figure 1 shows the Hofmeister series, representing a ranking of anions and cations in the order in which they influence macromolecular systems (Hochachka and Somero, 1984; Yancey, 1994). Ions to the left generally stabilize and enhance catalysis of macromolecular systems, while those to the right destabilize them. Those to the left promote "salting-out", while those to the right promote "salting-in" of proteins. Salting-out and salting-in refer to the capacities of these ions to either decrease, or increase, respectively, the solubilities of proteins (Hochachka and Somero, 1984; Yancey, 1994). Note that Na^+ and K^+ rank in the middle of the series, but that K^+ is less destabilizing than Na^+ . Effects of the anions and cations are algebraically additive, leading to counteraction if opposing ion types in the series are paired (Yancey, 1994).

			Stabilizing (Salting-Out)					Destabilizing (Salting-In)				
			←									→
Anions:	F ⁻	PO ₄ ³⁻	SO ₄ ²⁻	CH ₃ COO ⁻	Cl ⁻	Br ⁻	I ⁻	SCN ⁻				
Cations:	N ⁺ (CH ₃) ₄	N ⁺ (CH ₃) ₂ H ₂	N ⁺ H ₄	K ⁺	Na ⁺	Cs ⁺	Li ⁺	Mg ²⁺	Ca ²⁺	Ba ²⁺		

Figure 1. The Hofmeister (lyotropic) series of inorganic ions. Anions and cations are ranked in the order (from left to right) of their increasing destabilizing effects on proteins. The ions that are the most stabilizing tend to promote "salting-out" of proteins (Hochachka and Somero, 1984; Yancey, 1994).

The perturbing ions to the right of Figure 1 are destabilizing to proteins because they readily enter the hydration sphere of proteins and bind to non-specific sites (e.g. charged

protein groups), shifting the protein conformational equilibrium toward the unfolded state (Low, 1985; Yancey, 1994). The non-perturbing or "compatible" ions to the left of Figure 1 are stabilizing because they are preferentially excluded from the protein hydration domain shell. This preferential exclusion causes proteins to fold more compactly by aggregation, or precipitation (salting-out) (Low, 1985; Yancey, 1994).

Compatible solutes are solutes which mimic the Hofmeister stabilizing ions of Figure 1 by being preferentially excluded from the protein surface and its immediate hydration sphere, and which stabilize folded protein structures, promote subunit assembly and tend to promote salting-out (Low, 1985; Yancey, 1994). Structures of several compatible solutes accumulated by plants are illustrated in Figure 2. These include amino acids, methylated onium compounds, and polyols (acyclic and cyclic polyhydric alcohols [cyclitols]). The compounds shown in Figure 2 will be the focus of this review. However, these represent only a small fraction of the known compatible solutes in the plant kingdom. A large number of other methylated onium compounds (not shown in Fig. 2) have been identified in marine algae (Blunden and Gordon, 1986). The diversity of cyclic polyols found in higher plants is also far greater than depicted in Figure 2 (Popp and Smirnov, 1995). Because of space constraints we will not consider carbohydrates (e.g. sucrose, trehalose, raffinose, stachyose and fructans) which may play important roles as compatible molecules during desiccation and freezing.

The methylated quaternary ammonium and tertiary ammonium compounds of Figure 2 can be seen to be structural homologs of the stabilizing Hofmeister anion, $\text{N}^+(\text{CH}_3)_4$. All of the compatible solutes shown in Figure 2 share the properties of being highly soluble in water, and are electrically neutral in the physiological pH range. Generally they are non-inhibitory to enzymes at high concentrations (Wyn Jones *et al.*, 1977), they can block the inhibition of enzyme activity caused by perturbing solutes (Yancey, 1994), and they elevate the denaturation temperatures of proteins (Low, 1985; Yancey, 1994). Many of the compatible solutes listed in Figure 2 have been shown to stimulate the growth of salt-sensitive bacteria (e.g. *Escherichia coli* and *Salmonella typhimurium*) in highly saline media ($> 0.6 \text{ M NaCl}$) (Strøm *et al.*, 1983; Hanson *et al.*, 1991; 1994 a). The growth stimulation properties of these compatible solutes is often referred to as "osmoprotection" (Strøm *et al.*, 1983; Hanson *et al.*, 1994 a). The term "osmoprotectant" is therefore commonly applied to many of these compounds (Hanson and Burnet, 1994; McNeil *et al.*, 1999; Nuccio *et al.*, 1999). When provided at low concentrations ($< 1 \text{ mM}$), compounds such as glycine betaine and proline are accumulated to near 1 M concentrations in the bacterial cytoplasm, facilitating Na^+ and Cl^- exclusion and replacement of K^+ and glutamate, which serve as the principal osmolytes in the absence of an exogenous supply of compatible solute (Cayley *et al.*, 1991; 1992). This replacement of K^+ /glutamate restores cell water content and growth (Cayley *et al.*, 1991; 1992).

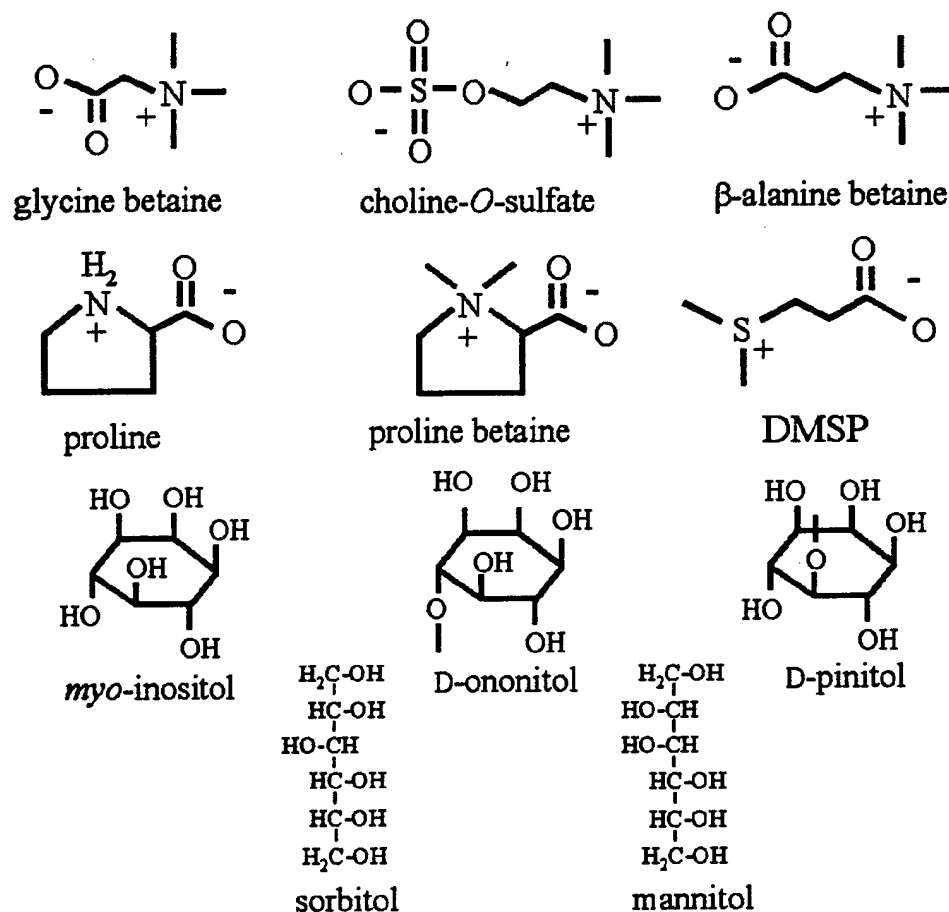


Figure 2. Structures of some compatible osmolytes found in plants. DMSP, 3-dimethylsulfoniopropionate.

Two explanations have been offered for the mechanism of compatible solute preferential exclusion from protein surfaces (Low, 1985; Yancey, 1994). First, many (but not all) of the solutes raise the surface tension of water, perhaps increasing the cohesive forces within the water structure; thus, they are proposed to be water structure-makers, enhancing water-water interactions (Low, 1985; Yancey, 1994). They either enhance the existing structure of water or perhaps reorganize the bulk water to a lesser degree at their molecular surfaces than structure-breaking solutes (Low, 1985). Second, they may be sterically excluded from the protein surface (Low, 1985; Yancey, 1994). Because compatible solutes take up more space than a water molecule, they are less able to pack next to a protein than water. Bulky solutes will increase protein activity by "crowding" proteins into a smaller space (Yancey, 1994). Osmolytes with -OH groups that could

form hydrogen bonds with proteins may have a poorer geometrical fit than water in the hydration lattice around the protein (Yancey, 1994).

In evolutionary terms the compatible solute accumulation strategy of adaptation to saline environments has key advantages over accumulation of cheaper, non-compatible, perturbing solutes, such as Na^+ and Cl^- , in the cytoplasm. Accumulation of perturbing solutes would require evolution of a large number of salt-tolerant enzymes, requiring substantial changes in protein amino acid composition to offset general Na^+ and Cl^- destabilization phenomena (Hochachka and Somero, 1984). Accumulation of a compatible solute for cytoplasmic osmoregulation would require far fewer changes, e.g. modification of the regulatory mechanisms controlling biosynthesis or catabolism of a non-perturbing compound (Hochachka and Somero, 1984). There is therefore great interest in identifying genes encoding key enzymes of compatible solute synthesis and/or catabolism because these genes hold promise for improving crop resistance to osmotic stresses (Strøm et al., 1983; Bohert and Jensen, 1996; Bohnert and Shen, 1999; Jain and Selvaraj, 1997; McNeil et al., 1999; Nuccio et al., 1999). In subsequent sections we will highlight recent progress in pathway characterization, gene identification and metabolic engineering of compatible solute accumulation in higher plants.

10.3. Pathways of synthesis and catabolism of compatible solutes found in plants

10.3.1. PROLINE

The imino acid proline is accumulated in the leaves of many halophytic plants from diverse families in response to salinity stress (Stewart and Lee, 1974; Treichel, 1975; Wyn Jones et al., 1977; Briens and Larher, 1982; Stewart and Larher, 1980). Proline is derived primarily from glutamic acid in reactions catalyzed by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and Δ^1 -pyrroline-5-carboxylate reductase (P5CR) (Fig. 3) (Verma and Zhang, 1998). P5CS is a bifunctional enzyme that phosphorylates glutamate in the 5- (γ -) position to form γ -glutamyl phosphate, and then reduces this labile intermediate to glutamic-5-semialdehyde (GSA) (Hu et al., 1992). GSA spontaneously cyclizes (with loss of water) to Δ^1 -pyrroline-5-carboxylate (P5C) (Fig. 3). P5C is subsequently reduced to proline in the reaction catalyzed by P5CR (Verma and Zhang, 1998). P5CS is strongly feedback inhibited by proline (Verma and Zhang, 1998). Both enzymes of proline biosynthesis are localized in the cytosol. This contrasts with the proline catabolism pathway (catalyzed by proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH)), which is mitochondrial (reviewed by Hare and Cress (1997)) (Fig. 3). Control of proline accumulation in response to salinity stress involves both increased synthesis and decreased proline oxidation, mediated (at least in some plant species) at the level of enhanced gene transcription of P5CS (Igarashi et al., 1997; Yoshida et al., 1995; Strizhov et al., 1997) and P5CR (Williamson and Slocum, 1992; Verbruggen et al., 1993), and concomitant down-regulation of PDH gene expression

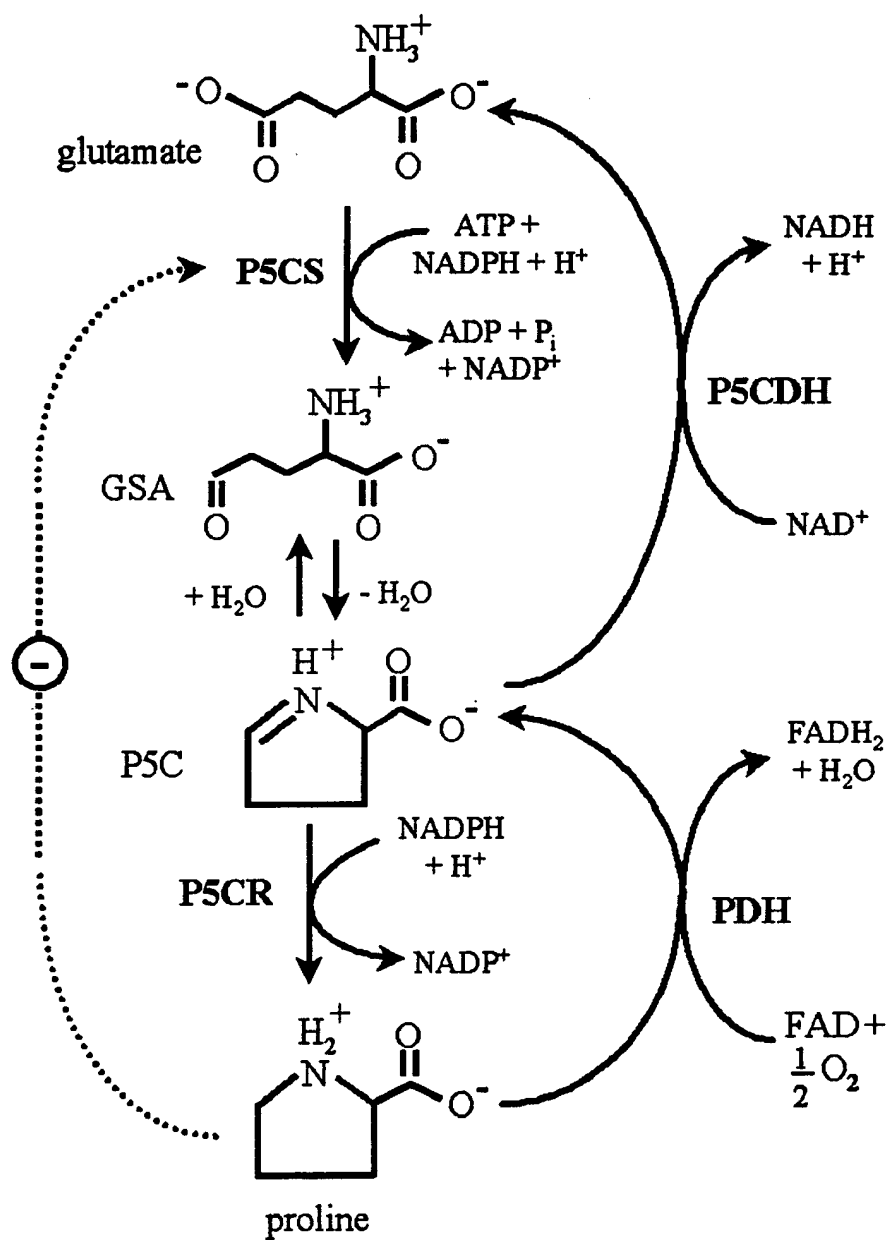


Figure 3. Pathways of synthesis and oxidation of proline in plants. GSA, glutamic-5-semialdehyde; P5C, Δ^1 -pyrroline-5-carboxylate; P5CDH, Δ^1 -pyrroline-5-carboxylate dehydrogenase; P5CR, Δ^1 -pyrroline-5-carboxylate reductase; P5CS, Δ^1 -pyrroline-5-carboxylate synthetase. The dotted line denotes feedback inhibition of P5CS by proline.

(Kiyosue *et al.*, 1996; Peng *et al.*, 1996; Yoshida *et al.*, 1997). Whereas PDH is normally induced by proline, this induction does not occur under osmotic stress (Peng *et al.*, 1996). It is noteworthy, however, that in tomato cell cultures adapted to salinity stress (15 g NaCl/L) a 30-fold increase in proline level occurs without any notable change in P5CS mRNA abundance (Fujita *et al.*, 1998). Ketchum *et al.* (1991) conclude from inhibitor studies with suspension cells of the halophytic grass, *Distichlis spicata*, that mRNA translation but not transcription is necessary for salinity stress induced proline accumulation, implying a post-transcriptional control mechanism(s).

Over-expression of P5CS in tobacco leads to proline accumulation and increased resistance to salinity stress (Kishor *et al.*, 1995). It is not clear whether the enhanced salt resistance of these transgenic plants can be ascribed solely to proline acting as a cytoplasmic compatible solute. Proline can also function as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989), and may play a role in the transport of reducing equivalents between cells and intracellular compartments (i.e. between cytoplasm and mitochondrion) (Hare and Cress, 1997).

10.3.2. GLYCINE BETAINE

Glycine betaine is widely distributed in the plant kingdom (Wyn Jones and Storey, 1981). It is accumulated to osmotically significant levels by many halophytic higher plants, including members of the Chenopodiaceae and salt-tolerant species of the Poaceae (Rhodes and Hanson, 1993). In chenopods, glycine betaine is synthesized from choline in reactions catalyzed by ferredoxin (Fd)-dependent choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) (Fig. 4A). Both CMO and BADH are predominantly localized in the chloroplast stroma of chenopods (Weigel *et al.*, 1986; 1988; Brouquisse *et al.*, 1989). Glycine betaine is also localized in the chloroplasts of salinized spinach leaves, where it provides osmotic adjustment (Robinson and Jones, 1986), and may play a key role in protection of the photosynthetic apparatus, particularly photosystem II (Murata *et al.*, 1992; Papageorgiou *et al.*, 1991; Homann, 1992; Williams and Gounaris, 1992; Mohanty *et al.*, 1993; Papageorgiou and Murata, 1995).

Both CMO (Burnet *et al.*, 1995) and BADH (Weretilnyk and Hanson, 1989) have been purified to homogeneity from spinach. cDNAs encoding BADH have been isolated from spinach (Weretilnyk and Hanson, 1990; Rathinasabapathi *et al.*, 1994) and sugar beet (McCue and Hanson, 1992 b). A cDNA encoding CMO has also been cloned from spinach (Rathinasabapathi *et al.*, 1997). The cDNA sequence confirms that CMO is an Fe-S protein and that CMO has a typical chloroplast transit peptide sequence.

BADH may be a relatively non-specific aldehyde dehydrogenase, acting on other aldehyde substrates in addition to betaine aldehyde (Trossat *et al.*, 1997). Thus, BADH will utilize 3-dimethylsulfoniopropionaldehyde, an intermediate in 3-dimethylsulfoniopropionate (DMSP) synthesis [see 10.3.6 below] and aldehydes (3-aminopropionaldehyde and 4-aminobutyraldehyde) involved in polyamine metabolism

(Trossat *et al.*, 1997). This may explain the occurrence of BADH in plants that do not accumulate glycine betaine (Ishitani *et al.*, 1993; Weretilnyk *et al.*, 1989), and in organs of glycine betaine-accumulating plants which do not contain glycine betaine (e.g. roots of cereals) (Ishitani *et al.*, 1995).

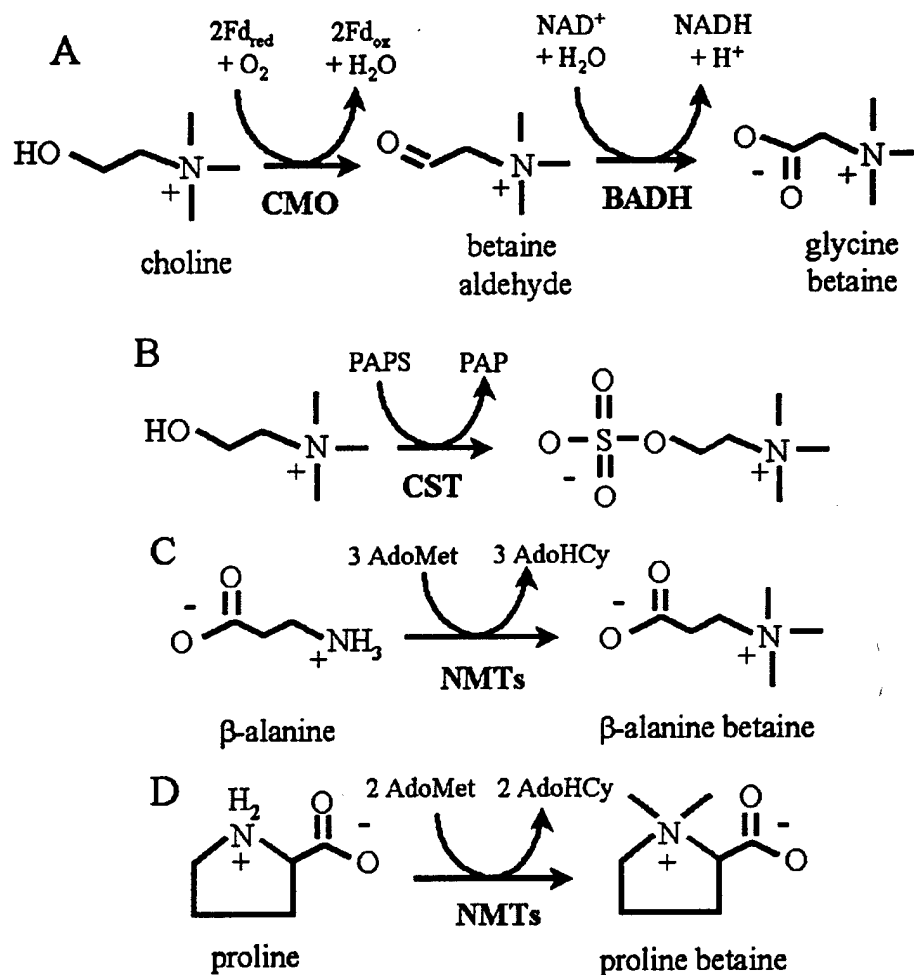


Figure 4. Pathways of synthesis of glycine betaine (A), choline-O-sulfate (B), β -alanine betaine (C), and proline betaine (D). AdoHCy, *S*-adenosyl-homocysteine; AdoMet, *S*-adenosylmethionine; BADH, betaine aldehyde dehydrogenase; CMO, choline monooxygenase; CST, choline sulfotransferase; Fd, ferredoxin; NMTs, *N*-methyltransferases; PAP, 3'-phosphoadenosine-5'-phosphate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate.

Salinity stress leads to two- to three-fold increases of CMO and BADH gene expression, and concomitant increases in enzyme level in spinach, sugar beet, and amaranth

(Weretilnyk and Hanson, 1989; 1990; Hanson *et al.*, 1995; Russell *et al.*, 1998). Abscissic acid (ABA) is implicated in the induction of BADH in cereals (Ishitani *et al.*, 1985). The signal causing induction of BADH in sugar beet does not appear to be either turgor reduction, NaCl, or ABA, but rather an unidentified biochemical signal translocated from roots to leaves in salinized plants (McCue and Hanson, 1992 b). Note that unlike proline, glycine betaine is not actively catabolized, so that control of glycine betaine concentration resides primarily at the level of synthesis rate and the rate of pool dilution with growth (Rhodes and Hanson, 1993).

In both maize and sorghum a number of naturally occurring glycine betaine-deficient inbred lines have been identified (Brunk *et al.*, 1989; Grote *et al.*, 1994). Glycine betaine-deficient maize lines lack the ability to accumulate glycine betaine in leaf tissue in response to either salinity stress or water deficits, due to a single recessive gene (*bet1*) (Yang *et al.*, 1995). Homozygous *bet1/bet1* maize lines are more salt-sensitive than near-isogenic homozygous *Bet1/Bet1* lines (Saneoka *et al.*, 1995), and exhibit greater membrane injury and damage to photosystem II in response to heat stress (Yang *et al.*, 1996). Glycine betaine-deficient maize lines lack the capacity to metabolize ^{14}C -choline to ^{14}C -glycine betaine, but are unimpaired in their ability to metabolize $^2\text{H}_3$ -betaine aldehyde to $^2\text{H}_3$ -glycine betaine, suggesting a lesion at the CMO step [or equivalent choline oxidizing activity] in the glycine betaine biosynthetic pathway (Lerma *et al.*, 1991). Because BADH appears to be localized in peroxisomes of monocotyledonous plants (Nakamura *et al.*, 1997), this raises the question of whether the subcellular localization [and mechanism] of choline oxidation is the same in grasses as in chenopods. Whereas chenopods derive choline destined for oxidation to glycine betaine predominantly from phosphocholine, via the action of a phosphocholine phosphatase (Hanson and Rhodes, 1981; Hanson *et al.*, 1995), grasses obtain choline primarily from phosphatidylcholine (Hitz *et al.*, 1981), presumably via the action of phospholipase D (Rhodes and Hanson, 1993).

In bacteria such as *Escherichia coli*, choline is an osmoprotectant only because it is oxidized to glycine betaine. The choline-glycine betaine pathway of *E. coli* consists of several genes encoding the functions of choline transport (*betT*), choline dehydrogenase (*betA*), betaine aldehyde dehydrogenase (*betB*), and a regulatory locus (*betI*) encoding a repressor protein (Lamark *et al.*, 1991; 1996). The entire *E. coli bet* gene cluster has been introduced into the freshwater cyanobacterium, *Synechococcus*, and this confers glycine betaine accumulation (60 to 80 mM) and increased salt tolerance, due in part to stabilization of photosystems I and II (Nomura *et al.*, 1995), and protection of ribulose 1,5-bisphosphate carboxylase/oxygenase from inactivation (Nomura *et al.*, 1998). However, this increased salt tolerance appears to depend on the presence of an exogenous supply of choline (Nomura *et al.*, 1995).

Because tobacco, rice and *Arabidopsis* are non-glycine betaine accumulators, these have been the organisms of choice for preliminary metabolic engineering experiments to test whether introduction of a capacity to synthesize glycine betaine influences higher plant salt tolerance (Hayashi and Murata, 1998; Takabe *et al.*, 1998; Nuccio *et al.*, 1998;

McNeil *et al.*, 1999). Transgenic tobacco plants expressing the *E. coli betA* gene encoding choline dehydrogenase, putatively capable of oxidizing both choline and betaine aldehyde, are reported to have increased salt tolerance (Lilius *et al.*, 1996), but because glycine betaine was not measured in these plants it is uncertain whether the salt tolerance phenotype can be attributed to glycine betaine accumulation. Transgenic rice plants expressing the *E. coli betA* gene are reported to accumulate up to 5 $\mu\text{mol/g}$ fresh weight of glycine betaine associated with increased salt tolerance (Takabe *et al.*, 1998). Introduction of the choline oxidase (*codA*) gene from *Arthrobacter* spp. into *Arabidopsis thaliana*, targeted to the chloroplast, leads to modest glycine betaine accumulation (1 $\mu\text{mol/g}$ fresh weight) [approx. 50 mM if assumed to be confined solely to the chloroplast], and enhanced salt and cold tolerance associated with protection of photosystem II (Hayashi *et al.*, 1997; Hayashi and Murata, 1998). The *codA* gene also appears to confer increased heat and light stress tolerance in *Arabidopsis* (Alia *et al.*, 1998; 1999). The *codA* gene has also been expressed in either the chloroplast or cytosol of rice (Sakamoto *et al.*, 1998). Chloroplastic expression appears to confer greater tolerance to photoinhibition under salinity and low temperature stress (Sakamoto *et al.*, 1998). However, expression of the cDNA encoding spinach CMO in the chloroplasts of tobacco leads to glycine betaine accumulation to levels of only a few percent of those found in natural glycine betaine accumulators (Nuccio *et al.*, 1998). Detailed analyses of choline metabolism in these transgenic tobacco plants suggests that the supply of choline may be limiting (Nuccio *et al.*, 1998; 1999). In tobacco, cytosolic choline is channeled almost exclusively into phosphatidylcholine via phosphocholine, making it difficult to divert choline to glycine betaine (Nuccio *et al.*, 1998). Glycine betaine accumulation places large demands on the synthesis of choline moieties, and on methyl groups required to generate choline (Hanson *et al.*, 1995). The capacity to accumulate glycine betaine may depend on special adaptations in choline and methyl group biogenesis that are not expressed in non-accumulators (Hanson *et al.*, 1995; McNeil *et al.*, 1999). In tobacco, and other non-glycine betaine accumulators, choline synthesis may be constrained at the first step in methylation of phosphoethanolamine en route to phosphocholine, catalyzed by phosphoethanolamine *N*-methyltransferase (Nuccio *et al.*, 1998; 1999).

10.3.3. CHOLINE-O-SULFATE

All members of the Plumbaginaceae accumulate choline-*O*-sulfate (Hanson *et al.*, 1994 a). This compound is synthesized from choline by a salt stress-inducible choline sulfotransferase (Rivoal and Hanson, 1994) (Fig. 4B). Salt glands of members of the Plumbaginaceae can excrete chloride but not sulfate; conjugation of sulfate with choline may therefore be a mechanism of sulfate detoxification, converting a normally destabilizing anion to a compatible solute (Hanson *et al.*, 1994 a). Consistent with this, accumulation of choline-*O*-sulfate is enhanced when Cl^- is replaced with SO_4^{2-} in the salinization medium (Hanson *et al.*, 1991). Because choline-*O*-sulfate synthesis competes with glycine betaine synthesis for available choline, this may have contributed

to the evolution of alternative betaine biosynthesis pathways (β -alanine betaine or proline betaine) which draw on substrates other than choline (Hanson *et al.*, 1994 a).

10.3.4. β -ALANINE BETAINE

β -Alanine betaine is restricted to species of the Plumbaginaceae (Hanson *et al.*, 1994 a). It is synthesized by direct *N*-methylation of the amino acid β -alanine, catalyzed by at least two *S*-adenosylmethionine (AdoMet)-dependent *N*-methyltransferases (Hanson *et al.*, 1991; 1994 a) (Fig. 4C). Unlike glycine betaine, β -alanine betaine synthesis does not require O_2 (Hanson *et al.*, 1994 a). This pathway may therefore represent an adaptation to anoxic saline environments (Hanson *et al.*, 1994 a). The β -alanine betaine biosynthesis pathway is active in both leaves and roots of β -alanine betaine-accumulating *Limonium* species (Hanson *et al.*, 1991). However, the precise origin of β -alanine in the Plumbaginaceae is not known.

10.3.5. PROLINE BETAINE AND HYDROXYPROLINE BETAINES

Proline betaine is accumulated by many higher plant species from diverse families (Wyn Jones and Storey, 1981; Hanson *et al.*, 1994 a; Gorham, 1995; Nolte *et al.*, 1997). The synthesis of proline betaine is thought to involve *N*-methylation of proline, via the intermediate *N*-methylproline (hygric acid) (Essery *et al.*, 1962), presumably catalyzed by AdoMet-dependent methyltransferases (Fig. 4D) (Hanson *et al.*, 1994 a). However, these enzymes have not yet been characterized. Hydroxyproline betaine often accumulates with proline betaine (Hanson *et al.*, 1994 a; Nolte *et al.*, 1997). The proline and/or *N*-methylproline hydroxylase(s) putatively involved in hydroxyproline betaine synthesis have not yet been identified (Hanson *et al.*, 1994 a).

Proline betaine and hydroxyproline betaine accumulate at the expense of free proline (Hanson *et al.*, 1994 a; Nolte *et al.*, 1997). Because proline betaine is a more potent osmoprotectant than proline, this may confer increased osmotic stress resistance (Hanson *et al.*, 1994 a). Synthesis of proline betaine would be expected to both alleviate P5CS from feedback regulation by proline, and restrict proline oxidation and hence compatible solute catabolism, perhaps contributing to maintenance of a greater compatible solute concentration (Samaras *et al.*, 1995).

10.3.6. DIMETHYLSULFONIOPROPIONATE

The sulfonium analog of β -alanine betaine, 3-dimethylsulfoniopropionate (DMSP), is synthesized and accumulated to osmotically significant levels by many marine algae (see e.g. Blunden and Gordon, 1986; Dickson and Kirst, 1986), and by a few higher plant species from diverse families; Asteraceae (e.g. *Wollastonia biflora*), and Poaceae (some species of *Spartina* and *Saccharum*) (Paquet *et al.*, 1994; James *et al.*, 1995). DMSP is as effective as glycine betaine as an osmoprotectant for *E. coli* (Paquet *et al.*, 1994), and

The diagram illustrates the metabolic pathways of methionine and its connection to the glutathione cycle. The central cycle involves the conversion of methionine (Met) to S-adenosylmethionine (AdoMet) by the enzyme methionine adenosyltransferase (MAT), which is coupled with the conversion of 2-oxoglutarate to glutamate. AdoMet is then used by various methyltransferases (MTs) to methylate substrates, releasing S-adenosylhomocysteine (AdoHCy). AdoHCy is converted to homocysteine (Hcy) by homocysteine adenosyltransferase (HAT). Hcy is then remethylated to methionine by methionine synthase (MS), which uses N⁵,N¹⁰-methylen-THF as a methyl donor, converting it to N⁵,N¹⁰-methyl-THF. The glutathione cycle is shown on the right, where glutathione (GSH) is oxidized to glutathione disulfide (GS-SG) by glutathione S-transferase (GST) during detoxification. GS-SG is then reduced back to GSH by glutathione reductase (GR), which uses NADPH as a reducing agent, converting it to NADP⁺. The diagram also shows the conversion of GSH to γ-glutamyl-L-cysteinylglycine (γ-Glu-Cys-Gly) by γ-glutamylcystine synthetase (γ-GCS), which is coupled with the conversion of 2-oxoglutarate to glutamate. The γ-Glu-Cys-Gly is then converted to cysteine by γ-glutamylcystine aminohydrolase (γ-GCA), which releases glutamate. Cysteine is then used for the synthesis of glutathione by glutathione synthetase (GS), which is coupled with the conversion of 2-oxoglutarate to glutamate. The diagram also shows the conversion of glutamate to 2-oxoglutarate by 2-oxoglutarate aminotransferase (2-OGAT), which releases glutamate. The diagram is a schematic representation of the metabolic pathways and their interconnections.

Figure 5. Pathways of synthesis of 3-dimethylsulfoniopropionate (DMSP) in higher plants (left) and marine algae (right). For the higher plant pathway *Wb* denotes the pathway found in *Wollastonia biflora* (Asteraceae), and *Sa* the pathway in *Spartina alterniflora* (Poaceae). The question mark adjacent to *Wb* indicates that the precise mechanism of conversion of SMM to DMSP-aldehyde in *Wollastonia* is not known; it may involve transamination and decarboxylation. AdoHCy, S-adenosylhomocysteine; AdoMet, S-adenosyl-methionine; DADH, DMSP-aldehyde dehydrogenase; DMSHB, 4-dimethylsulfonio-2-hydroxybutyrate; DMSP-amine, 3-dimethylsulfoniopropylamine; DMSP-aldehyde, 3-dimethylsulfoniopropionaldehyde; MAT, methionine:2-oxoglutarate aminotransferase; Met, methionine; MMT, AdoMet:methionine S-methyltransferase; MOx, methionine oxidase; MTHB, 4-methylthio-2-hydroxybutyrate; MTHBMT, AdoMet:MTHB S-methyltransferase; MTOB, 4-methylthio-2-oxobutylate; MTOBR, MTOB reductase; SMM, S-methylmethionine.

In *Wollastonia*, DMSP is synthesized by methylation of methionine to *S*-methylmethionine (SMM) in the cytosol (Hanson *et al.* 1994 b; James *et al.* 1995 a,b), transport of SMM into the chloroplast, transamination or deamination and decarboxylation of SMM to DMSP-aldehyde (James *et al.*, 1995 b), and oxidation of the aldehyde to DMSP in the chloroplast (Trossat *et al.*, 1996; 1997) (Fig. 5). The first enzyme of the pathway, AdoMet:L-methionine *S*-methyltransferase (MMT) has been purified from *Wollastonia* leaves (James *et al.*, 1995 a), and its cDNA cloned (Bourgis *et al.*, 1999). The final step in the pathway is catalyzed by a chloroplast localized DMSP-aldehyde dehydrogenase (DADH), which may be identical to the BADH involved in glycine betaine synthesis (Trossat *et al.*, 1997; Vojtechová *et al.*, 1997). The enzyme(s) responsible for metabolism of SMM to DMSP-aldehyde in *Wollastonia* is not yet known. It is proposed that SMM is converted to DMSP-aldehyde via a specialized transaminase-decarboxylase (Rhodes *et al.*, 1997).

The pathway of DMSP synthesis in the salt marsh grass *Spartina alterniflora* is similar to that in *W. biflora* except with respect to the SMM \rightarrow DMSP-aldehyde steps in the pathway (Fig. 5). In *Spartina*, radiolabeling experiments with ^{35}S -methionine and ^{35}S -SMM show that DMSP-amine behaves as an intermediate, implicating SMM decarboxylation as the first step in the conversion of SMM to DMSP-aldehyde (Kocsis *et al.*, 1998). DMSP-amine may then be metabolized to the aldehyde via an amine oxidase, but this enzyme has not been characterized.

The pathway of DMSP synthesis in higher plants is completely different from that found in marine algae (Gage *et al.*, 1997). In marine algae methionine is first converted to the 2-keto acid, 4-methylthio-2-oxobutyrate (MTOB), via the action of either methionine:2-oxoglutarate aminotransferase or methionine oxidase (Gage *et al.*, 1997; Summers *et al.*, 1998) (Fig. 5). MTOB is then reduced to 4-methylthio-2-hydroxybutyrate (MTHB) by an NADPH-dependent reductase (Summers *et al.*, 1998). MTHB is subsequently *S*-methylated to 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) which is then oxidatively decarboxylated to DMSP (Gage *et al.*, 1997; Summers *et al.*, 1998) (Fig. 5). The latter enzyme has not been identified.

Because DMSP does not contain N, the accumulation of DMSP as a compatible solute could represent a special adaptation to N-limiting environments. N-deficiency increases DMSP levels in *Wollastonia* (Hanson *et al.*, 1994 b) and marine algae (Gage *et al.*, 1997; Dickson and Kirst, 1986).

10.3.7. POLYOLS

Like DMSP, polyhydric alcohols (polyols) also lack N, and so could represent important compatible solutes where synthesis of nitrogenous solutes (e.g. proline and/or betaines) is limited by N availability. The acyclic polyols, sorbitol and mannitol are accumulated by many marine algae, lower plants and species from diverse higher plant families, including certain halophytes (Ahmad *et al.*, 1979; Briens and Larher, 1982; 1983; Popp

and Smirnov, 1995). Sorbitol is accumulated to high levels (up to 70 mM on a tissue water basis) by members of the Plantaginaceae in response to salinity stress, while mannitol can reach concentrations as high as 200 mM on a tissue water basis in leaf and stem tissues of the mangrove species *Laguncularia racemosa* (Popp and Smirnov, 1995).

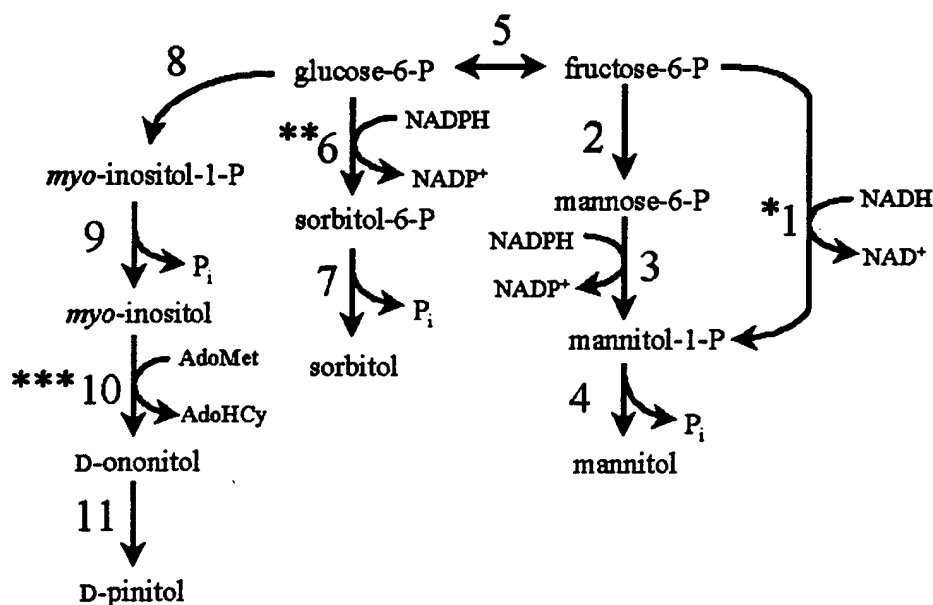


Figure 6. Pathways of synthesis of the acyclic polyols, mannitol and sorbitol, and the cyclic polyols, *myo*-inositol, D-ononitol, and D-pinitol. * Denotes genes used in metabolic engineering experiments with tobacco (see text for details). Enzymes (and genes) are as follows:

1. Mannitol-1-phosphate dehydrogenase,
* *mtlD* (*Escherichia coli*)
2. Mannose-6-phosphate isomerase.
3. Mannose-6-phosphate reductase.
4. Mannitol-1-phosphate phosphatase.
5. Hexose-phosphate isomerase.
6. Sorbitol-6-phosphate dehydrogenase [aldose-6-phosphate reductase (NADPH)],
** *S6pdh* (*Malus domestica*).
7. Sorbitol-6-phosphate phosphatase.
8. Glucose-6-phosphate cycloaldolase [*myo*-inositol-1-phosphate synthase].
9. *Myo*-inositol-1-phosphate phosphatase [inositol monophosphatase].
10. *Myo*-inositol-*O*-methyltransferase,
*** *Imt1* (*Mesembryanthemum crystallinum*).
11. Ononitol epimerase.

Figure 6 shows the pathways of synthesis of sorbitol, mannitol and the cyclic polyols (cyclitols), D-ononitol and D-pinitol. Note that sorbitol and mannitol draw directly from the glucose-6-phosphate and fructose-6-phosphate pools, and that sorbitol synthesis potentially competes with *myo*-inositol synthesis, and hence the synthesis of D-ononitol

and D-pinitol. The latter cyclic polyols are accumulated by many legumes in response to water deficits and salinity stress (Popp and Smirnov, 1995). D-Pinitol is also accumulated by the halophytic facultative crassulacean acid metabolism plant, *Mesembryanthemum crystallinum*, in response to salinization (Bohnert *et al.*, 1995; Bohnert and Jensen, 1996).

In the Rosaceae, and Apiaceae, sorbitol and mannitol, respectively, are utilized as phloem translocated photoassimilates (Locy, 1994; Popp and Smirnov, 1995). These polyols are actively catabolized in non-photosynthetic sink tissues by NAD⁺-dependent sorbitol and mannitol dehydrogenases (Locy, 1994; Williamson *et al.*, 1995). The mannitol dehydrogenase of celery has been cloned and has been shown to be repressed by salinity stress (Williamson *et al.*, 1995). In celery plants mannitol dehydrogenase activity and mannitol pool size are inversely correlated, suggesting that regulation of catabolism of mannitol is a major determinant of the tissue concentration of this compatible solute (Williamson *et al.*, 1995). However, little is known about the regulation of sorbitol and mannitol catabolism in members of the Plantaginaceae and mangroves that accumulate high levels of these solutes in response to salinity stress (Locy, 1994; Popp and Smirnov, 1995). The cyclic polyols, D-ononitol and D-pinitol, appear to be less rapidly catabolized (Popp and Smirnov, 1995), and control of tissue concentrations may therefore be primarily determined by synthesis rate.

In members of the Apiaceae (e.g. celery and celeriac), mannitol is synthesized from fructose-6-phosphate via mannose-6-phosphate via the action of mannose-6-phosphate isomerase and an NADPH-dependent mannose-6-phosphate reductase (M6PR) (Fig. 6). The gene encoding M6PR has been cloned from celery, and shows considerable sequence similarity to the sorbitol-6-phosphate dehydrogenase of apple (Everard *et al.*, 1997). M6PR is expressed in green tissues and is under tight transcriptional regulation during leaf development (Everard *et al.*, 1997).

Tobacco has served an excellent model system to explore the potential contribution of the acyclic and cyclic polyols. Wild-type tobacco lacks mannitol, sorbitol, D-ononitol and D-pinitol, but contains *myo*-inositol (Sheveleva *et al.*, 1997; 1998). Tobacco has been engineered to accumulate mannitol by introducing the mannitol-1-phosphate dehydrogenase gene (*mtlD*) from *Escherichia coli* (Tarczynski *et al.*, 1992; 1993). This enzyme converts fructose-6-phosphate to mannitol-1-phosphate which is then cleaved to mannitol, presumably by a constitutive, non-specific sugar alcohol-phosphate phosphatase (Fig. 6). Constitutive production of mannitol in tobacco has been achieved by expressing *mtlD* in either the cytosol (Tarczynski *et al.*, 1992; 1993) or chloroplast (Shen *et al.*, 1997 a,b); both result in increased salt tolerance. Expression of *mtlD* in the chloroplast results in accumulation of mannitol to concentrations of up to 100 mM in tobacco chloroplasts (Shen *et al.*, 1997 b). Mannitol may function not only as a compatible solute, but also as a hydroxyl radical scavenger in this compartment (Smirnov and Cumbes, 1989; Shen *et al.*, 1997 b).

In apple, sorbitol is derived from glucose-6-phosphate via the catalytic action of an NADPH-dependent sorbitol-6-phosphate dehydrogenase (Tao *et al.*, 1995), and sorbitol-6-phosphate phosphatase (Popp and Smirnov, 1995) (Fig. 6). Constitutive expression of the apple sorbitol-6-phosphate dehydrogenase in tobacco, leads to necrotic lesions in proportion to the size of the sorbitol pool accumulated [up to 130 mM on a whole tissue water basis] (Sheveleva *et al.*, 1998). Again, as with mannitol, it is presumed that sorbitol-6-phosphate is catabolized to sorbitol by a constitutive non-specific sugar alcohol-phosphate phosphatase in these transgenic tobacco plants (Sheveleva *et al.*, 1998). The autotoxicity of sorbitol accumulation in tobacco may be the result of depletion of *myo*-inositol (Sheveleva *et al.*, 1998), although toxic accumulation of sorbitol-6-phosphate cannot yet be ruled out. Because of the growth inhibition caused by overproduction of sorbitol in transgenic tobacco, it has not been possible to accurately assess the contribution of this solute to salt tolerance of tobacco (Sheveleva *et al.*, 1998).

Myo-inositol is derived from glucose-6-phosphate via the action of glucose-6-phosphate cycloaldolase [*myo*-onositol-1-phosphate synthase] and *myo*-inositol-1-phosphate phosphatase (Fig. 6). D-Ononitol is then formed by *O*-methylation of *myo*-inositol, catalyzed by an AdoMet-dependent *myo*-inositol *O*-methyltransferase (Vernon and Bohnert, 1992). D-Pinitol is derived from D-ononitol via the action of an ononitol epimerase (Bohnert and Jensen, 1996) (Fig. 6). In *M. crystallinum* the entire pathway is salinity stress inducible (Vernon and Bohnert, 1992; Bohnert and Jensen, 1996). *Myo*-inositol may not only serve as a substrate for the production of compatible solutes but also as a leaf-to-root signal that promotes sodium uptake in *M. crystallinum* (Nelson *et al.*, 1999). Note that wild-type tobacco has the capacity to synthesize and accumulate *myo*-inositol in response to salinity and drought stress, but lacks the terminal methyltransferase and epimerase of the pathway (Sheveleva *et al.*, 1997). Sheveleva *et al.* (1997) have constitutively expressed the *M. crystallinum* gene encoding *myo*-inositol-*O*-methyltransferase in tobacco. This confers stress-induced D-ononitol accumulation [up to 35 mM on a whole tissue water basis] and increased salt tolerance (Sheveleva *et al.*, 1997). Unlike constitutive sorbitol accumulation, stress-inducible D-ononitol accumulation does not result in necrotic lesions (Sheveleva *et al.*, 1997).

10.4. Evidence for cytoplasmic localization of compatible solutes

Central to the compatible solute hypothesis is that these solutes are accumulated in the cytosol and/or cytoplasmic organelles such as chloroplasts. Approaches to testing this fundamental premise of this hypothesis have been to isolate intact vacuoles and chloroplasts from salinized plant tissues, demonstrating that the ratios of compatible solutes to inorganic ions in these compartments are substantially different from those of whole tissue extracts (Leigh *et al.*, 1981; Wyn Jones *et al.*, 1977; Robinson and Jones, 1986). However, considerable technical difficulties are associated with correcting for solute leakage from organelles during aqueous extraction (see e.g. Leigh *et al.*, 1981; Robinson and Jones, 1986).

Using organelle isolation methods evidence for a predominantly cytoplasmic (extra-vacuolar) localization of proline has been obtained for red beet cells (Leigh *et al.*, 1981) and *Distichlis spicata* cell cultures (Ketchum *et al.*, 1991). The cytoplasmic concentration of proline in 200 mM NaCl-grown *Distichlis* cells has been determined to be within the range 237 - 311 mM; approximately 10-times the concentration in the cell as a whole (Ketchum *et al.*, 1991).

Robinson and Jones (1986) have determined that the glycine betaine concentration of chloroplasts isolated from leaves of spinach plants salinized to 200 mM NaCl, can approach 300 mM, contributing at least one-third of the total osmotic potential of the chloroplast. This is consistent with other investigations of the compartmentation of glycine betaine in chenopods which have demonstrated that this solute is extra-vacuolar (Wyn Jones *et al.*, 1977; Hall *et al.*, 1978; Leigh *et al.*, 1981; Matoh *et al.*, 1987) or chloroplastic (Schröppel-Meier and Kaiser, 1988; Genard *et al.*, 1991). It is not known whether glycine betaine is localized in chloroplasts of grasses which accumulate this solute. The intracellular localizations of choline-*O*-sulfate, β -alanine betaine, proline betaine and hydroxyproline betaine in the Plumbaginaceae have not been investigated. However, there is strong circumstantial evidence that these solutes have replaced the function of glycine betaine as cytoplasmic compatible solutes. Thus, the total quaternary ammonium compound levels of contrasting glycine betaine and β -alanine betaine accumulating *Limonium* species are both tightly correlated with total solute potential (at 100% relative water content) in leaf tissues of plants grown in nutrient solutions containing 0 - 600 mM NaCl (Hanson *et al.*, 1991).

Consistent with the chloroplastic localization of the terminal enzymes of the DMSP synthesis pathway in *Wallastonia biflora*, DMSP is predominantly localized in the chloroplasts of salinized *W. biflora* plants, where its concentration may exceed 130 mM (Trossat *et al.*, 1998).

Pinitol concentrations in the chloroplasts isolated from leaves of *M. crystallinum* plants have been estimated to be 230 mM, whereas the cytosol concentration was estimated to be 100 mM [cf. 10 mM on a tissue sap basis] (Paul and Cockburn, 1989). Pinitol was not detected in vacuoles (Paul and Cockburn, 1989). However, as discussed by Popp and Smirnoff (1995) the concentrations of certain cyclitols in the Fabaceae and mangroves can exceed 200 mM on a tissue water basis and it seems highly unlikely that these could be exclusively localized in the cytoplasm in these species. Vacuolar accumulation of polyols or other carbohydrates may provide osmotic adjustment in saline environments when Na^+ and Cl^- are excluded from leaf tissues (Briens and Larher, 1982). Thus, Briens and Larher (1982) have noted an inverse correlation between leaf inorganic ion content and organic solute concentration among diverse halophytic plant species.

10.5. Concluding discussion

It has long been recognized that salt-tolerant and salt-sensitive plant species differ dramatically with respect to their accumulation of organic solutes, particularly in response to salinity stress (Wyn Jones and Storey, 1981; Blunden and Gordon, 1986; Gorham, 1995; Smirnov and Stewart, 1985; Rhodes and Hanson, 1993; Popp and Smirnov, 1995; Bohnert and Jensen, 1996). Because these organic solutes/osmolytes are non-toxic to enzyme systems across a wide concentration range, and there is sparse but growing evidence that these solutes are accumulated in the cytoplasm, it seems likely that these solutes play a central role in cytoplasmic osmotic adjustment by acting as "compatible cytoplasmic solutes" in plants (Wyn Jones *et al.* 1977), as in other organisms (Hochachka and Somero, 1984; Yancey, 1994). According to Hochachka and Somero (1984) the selection of a particular organic solute for use as an osmotic agent is subsidiary to the fundamental "design principle of establishing a microenvironment for macromolecules in which their structural and functional properties are optimized for catalysis, metabolic regulation, information transfer, and mechanical work, and in which a balance is achieved between stability and instability of structure." Because many different organic compounds can satisfy this microenvironmental design principle, we should not be surprised by the great diversity of the metabolic pathways of plant osmolyte synthesis discussed above.

Recent studies with transgenic glycophytic plants engineered for the synthesis and accumulation of certain of these compounds (including proline, glycine betaine, mannitol and D-ononitol) indicate that such accumulation may confer modest increases in salt tolerance, and in some cases, increased resistance/tolerance to other stresses (drought, cold, heat or light). In the case of mannitol accumulation it appears that the mode of action of this solute may not be solely in cytoplasmic osmotic adjustment; mannitol may play a more significant role as a hydroxyl radical scavenger in chloroplasts (Shen *et al.*, 1997 b). These advances with model glycophytes, achieved by manipulating single genes, hold substantial promise for designing and implementing engineering strategies to enhance salinity stress resistance of crop plants (Bohnert and Jensen, 1996; Bohnert and Shen, 1999; Jain and Selvaraj, 1997). However, these preliminary genetic interventions have also shown that: (i) the introduction of a foreign gene conferring synthesis of a solute may substantially disturb normal metabolism and growth (Sheveleva *et al.*, 1998; Serrano *et al.*, 1999), and (ii) the engineered gene may often be embedded in a rigid metabolic network that resists change and restricts solute accumulation (Nuccio *et al.*, 1998, 1999). Hare *et al.* (1998) suggest that disturbance of hexose sensing may be an important contributing factor to the stress-tolerance phenotypes of plants engineered for carbohydrate osmolytes, and that there is a need for re-assessment of the significance of proline and glycine betaine accumulation as mechanisms for buffering cellular redox potential. Further work is needed to identify the unique regulatory features of metabolism that permit the accumulation of substantial levels of compatible solutes found in nature (Jain and Selvaraj, 1997), and to combine these traits with other equally important osmoregulatory features of halophytes discussed in this volume, including potassium/sodium discrimination, sodium exclusion, and sodium compartmentation in the vacuole.

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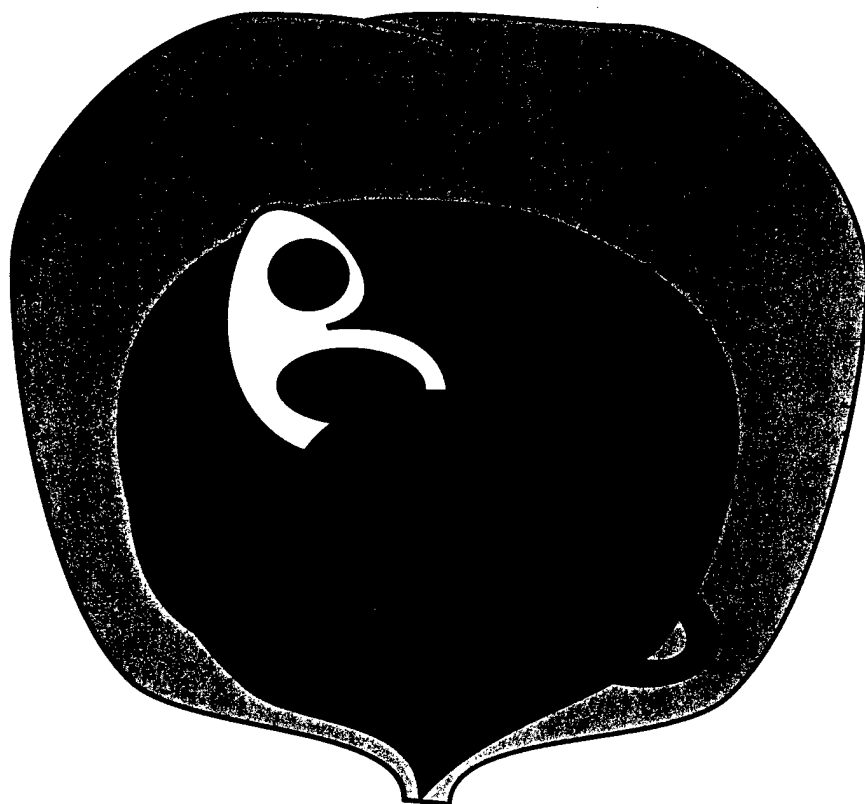
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Metabolic Networking in Plants



April 22-25, 1999

**The 1st annual
Plant Biochemistry and Molecular
Biology Conference**

Sponsored by
National Science Foundation
U.S. Department of Agriculture

and co-sponsored by Department of Molecular Biology
Department of Botany
Texas State University

Program

Thursday, April 22

- 2:00 - 4:00 Registration (2nd floor lobby)
- 2:00 - 4:00 Reception (2nd floor lobby)

Plenary Session I

Eve Syrkin Wurtele, Session Chair

- 4:00 - 4:10 Welcome: Dean Patricia Swan
- 4:10 - 4:55 **Coruzzi**, *Amino acid signaling and sensing in Arabidopsis*
- 4:55 - 5:40 **Stitt**, *Coordinate regulation in central metabolic pathways*
- 5:40 - 7:20 Dinner (Stagedoor Cafe), Jack Gallup, guitar

Friday, April 23

- 8:30 - 9:00 Continental Breakfast (1st floor lobby)

Plenary Session II

Martha G. James, Session Chair

- 9:00 - 9:45 **Oliver**, *Glutathione synthesis in plants: Multiple controls in response to multiple stresses*
- 9:45 - 10:30 **Chapple**, *Secondary metabolism in Arabidopsis: biochemistry and biotechnology*
- 10:30 - 11:00 Break with refreshments (1st floor lobby)
- 11:00 - 12:15 **Presented Papers - Session I**
- 12:15 - 2:00 Lunch (Stagedoor Cafe)

Plenary Session III

John P. Davies, Session Chair

- 2:00 - 2:45 **Nikolau**, *Molecular biology of acetyl-CoA generation and metabolism in plants*
- 2:45 - 3:30 **Browse**, *Arabidopsis mutants reveal many roles for lipid metabolism in plants*
- 3:30 - 4:30 **Poster Session I** and coffee
- 4:30 - 5:15 **Bush**, *Resource allocation and multicellular growth: Molecular analysis of plant sugar and amino acid transporters*
- 5:15 - 6:00 **Leustek**, *Environmental factors that regulate sulfur assimilation in plants*
- 6:00 - 6:15 Group photo
- 6:15 - 7:45 Dinner (Stagedoor Cafe), Paula Helmuth, piano
- 7:45 - 9:30 Dessert Party, Eve Syrkin Wurtele's home (3425 Oakland Street)

Saturday, April 24

8:00 - 8:30 Continental breakfast (1st floor lobby)

Plenary Session IV Martin H. Spalding, Session Chair

8:30 - 9:15 **Sheen**, *Sugar and hormonal signaling network*

9:15 - 10:00 **Rhodes**, *Computer simulation of isotope labeling kinetics of metabolic networks: Uses in interpreting precursor-product relationships, fluxes and compartmentation of pools*

10:00 - 10:30 Break with refreshments (1st floor lobby)

10:30 - 12:15 **Presented Papers** - Session II

12:15 - 1:45 Lunch (Stagedoor Cafe)

Plenary Session V Basil J. Nikolau, Session Chair

1:45 - 2:30 **Pichersky**, *Pathways that lead to the synthesis of scent and flavor compounds*

2:30 - 3:00 **Presented Paper** - Session III: Computational Biology

3:00 - 4:45 **Poster Session II** with coffee

4:45 - 5:30 **Shanklin**, *Encouraging enzymes to do what we want them to do: Rational and irrational design*

5:30 - 6:15 **Murata**, CARVER LECTURER, *Metabolic feedback in the fatty-acid desaturation of membrane lipids*

6:15 - 8:00 Dinner (Stagedoor Cafe), Mary Foss, harp

Sunday, April 25

8:00 - 9:00 Continental breakfast (1st floor lobby)

Plenary Session VI Parag R. Chitnis, Session Chair

9:00 - 9:30 **Presented Papers** - Session IV

9:30 - 10:15 **Grotewold**, *Plant Myb-domain proteins and metabolic diversity: Tools for engineering secondary metabolism?*

10:15 - 10:40 Break with refreshments (1st floor lobby)

10:40 - 11:25 **Bohnert**, *Metabolic engineering of plant abiotic stress tolerance*

11:35 - 12:10 Buses leave for the airport

COMPUTER SIMULATION OF ISOTOPE LABELING KINETICS OF METABOLIC NETWORKS: USES IN INTERPRETING PRECURSOR-PRODUCT RELATIONSHIPS, FLUXES AND COMPARTMENTATION OF POOLS

David Rhodes, Purdue University; Yair Shachar-Hill, New Mexico State University; Scott D. McNeil & Andrew D. Hanson, University of Florida

Interactive computer models for interpreting *in vivo* isotopic tracer data will be described and discussed. Simplified hypothetical examples will first be employed to introduce modeling principles – the deduction of precursor-product relationships, fluxes and compartmentation of metabolite pools from carefully timed *in vivo* isotope labeling data and pool size measurements. These principles will be applied to analysis of the pathways of synthesis of the osmolyte 3-dimethylsulfoniopropionate (DMSP) in the marine alga, *Enteromorpha intestinalis*, and the higher plant, *Spartina alterniflora*. Both organisms synthesize DMSP from methionine, but employ different intermediates. Computer modeling of ^{35}S tracer data demonstrates that in *Enteromorpha* DMSP synthesis proceeds via the steps methionine \rightarrow 4-methylthio-2-hydroxybutyrate \rightarrow 4-dimethylsulfonio-2-hydroxybutyrate \rightarrow DMSP (Gage et al., 1997), whereas in *Spartina* the pathway proceeds via the steps methionine \rightarrow S-methylmethionine \rightarrow 3-dimethylsulfoniopropylamine \rightarrow DMSP (Kocsis et al., 1998). In both cases computer-assisted analysis of the pathways permit estimates to be made of the fluxes to DMSP, the sizes of metabolically active and relatively inactive (storage) pools of intermediates, and the transport rates between them.

We then extend these models to consider the network of choline metabolism in tobacco leaves, with emphasis on identifying the metabolic constraints that limit the synthesis and accumulation of the osmolyte, glycine betaine, in transgenic tobacco expressing ferredoxin-dependent choline monooxygenase (CMO) in the chloroplast (Nuccio et al., 1998). Modeling of [^{14}C]-choline metabolism (including phosphocholine and phosphatidylcholine synthesis and catabolism) suggests that the primary constraint is a low affinity of the chloroplast choline transport system. The latter does not compete effectively with the high affinity choline kinase and tonoplast choline transport system for available cytosolic choline. The models reveal other constraints on glycine betaine synthesis that may help guide future rounds of metabolic engineering to enhance glycine betaine synthesis. Greatly elevating flux to glycine betaine is likely to occur at the expense of flux to phosphatidylcholine, with potentially growth inhibitory consequences unless the synthesis of choline moieties is also enhanced. This example illustrates how mathematical models can help build understanding of the complex interactions of metabolic networks which often confound metabolic engineering efforts to redirect fluxes (Bailey, 1998).

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for additional information on computer simulation of metabolism and examples of interactive, online models see <http://www.hort.purdue.edu/cfpesp/models/models.htm>